Effects of Asbestos in the Human Respiratory Tract

Thesis

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Abstract

The aims of this investigation were to study differences in the protein composition and cellular content of the sputum from workers in an asbestos textile factory and also to reconstruct the sequence of events that occurs when inhaled chrysotile is retained in the human respiratory tract. The mean value of total protein in the sputum from workers exposed to chrysotile without asbestosis was lower than that from workers in the unexposed and the asbestotic groups. Within the group 'exposed without asbestosis' sputum total protein was correlated with all electrophoretic fractions except beta/gamma globulin whereas within the group 'exposed with asbestosis' sputum protein levels were correlated only with beta/gamma globulin. Immunoanalysis showed increased transferrin in sputa from exposed donors but no specific marker protein for asbestosis was found. A non-serous, PAS reacting protein with $beta_3$ electromobility was isolated by cation exchange separation from all sputa examined.

Cytological analysis showed that half of the exposed donors possessed Type 1 pneumocytes in their sputa; Type 2 pneumocytes and iron-containing histiocytes were also found but with lower frequency. Histiocytes with endocytosed chrysotile or asbestos bodies were found only in sputa from asbestotic donors and extracellular asbestos bodies were found five times more frequently in these sputa than in those from exposed donors without asbestosis. None of these findings were seen in sputa from unexposed donors. A negligible amount of water was taken up by chrysotile from

wet air at 37°C during the inhalation period, 1.74 seconds, but when chrysotile was immersed in sputum, water uptake preceeded protein adsorption. About 45 mg protein/g chrysotile was taken up in the proportion 1:1:3:1 of albumin: alpha globulin: beta/gamma globulin: lysozyme independent of sputum protein concentration and about two thirds of all adsorbed protein was PAS positive protein. When chrysotile was coated with sputum protein, its cytolytic property was markedly attenuated and viable histiocytes were still present after 25 hours exposure to the coated fibre.

The results indicate that some part of retained chrysotile reaches the lung alveoli causing loss of alveolar epithelial cells although in exposed persons without asbestosis the capillary endothelium probably remains intact.

Acknowledgements

This study was made possible by the cooperation of The Industrial Health Unit, B.B.A. Group p l c and by grants from the Asbestos Research Council (Medical Committee), B.B.A. Group p l c, Cleckheaton and the Chest Unit Research Fund, St. Luke's Hospital, Bradford.

I thank my supervisors, Dr. V.G. Daniels and Dr. J.N. Thomas, for their counselling and Professor T.G. Baker, School of Medical Sciences, University of Bradford and Dr. D.A.G. Newton, Consultant Physician, Bradford Hospitals for time and advice freely given.

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CHAPTER ONE

Introduction

1.1.1 Introduction

The mammalian respiratory system is poorly constructed to cope with microparticulates less than 200 μm diameter in respired air since it possesses no excretion mechanism for solids trapped in the distal airway. Evolutionary precursors of man lived in an aqueous environment where suspended solids pass through gill chambers and pose no excretion problem. Carter (1931), reviewing respiration in animals, considers that the evolutionary migration from water to land has occurred many times. He concludes that migration was probably motivated by fluctuations in water levels induced by tidal rhythms and seasonal drought in littoral areas and by the stagnation of water in shallow tropical bogs with consequent reduction in available oxygen. In these circumstances, an ability to utilise atmospheric oxygen had distinct advantages and it is probable that airborne dust was not a significant hazard at this time.

1.1.2 'Natural' Dust Hazards

Casual consideration might suggest that, prior to the use of metal hand tools and the subsequent industrialisation of societies, early man's exposure to dust occurred only during brief, intermittent periods of storm. However, since the conquest of fire, temperate-zone dwellers must have experienced protracted exposure to high concentrations of carbon in smoke during cold spells in confined and poorly-ventilated conditions. Also, nomadic tribes in arid desert regions have endured virtually continuous exposure to silica dust. It is interesting to speculate on possible adaptive

processes accruing from breathing such atmospheres.

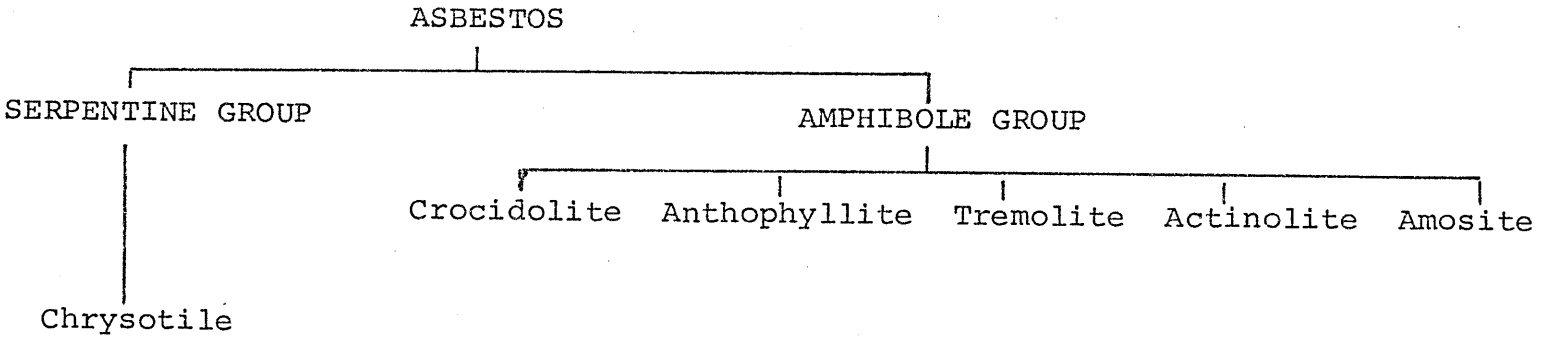
1.1.3 Industrial Dust

In the more recent past and especially since the Industrial Revolution, workers in many industries have been exposed to high concentrations of dust generated by factory conditions. The diseases that can develop in lungs as a consequence of dust inhalation and retention are collectively described as the 'pneumoconioses' a word coined by Zenker in 1867. Some of these dusts cause damage to the airways but sometimes the crippling factor is the development of fibrosis within the lung parenchyma causing reduction of the gas-transfer area often leading eventually to respiratory failure. The progressive fibrosis of the lungs is one possible sequel to the inhalation of chrysotile asbestos and the various events leading to the onset of fibrosis are presented in this study.

1.2.1 Uses of Asbestos

Man's use of asbestos can be traced back to early Greek civilisation (Hunter, 1974), and the word itself derives from that language literally meaning 'inextinguishable' indicating that it is a material not affected by fire. The mineral is useful because of its fibrous character and silky texture - properties that earned it the name 'stone flax' - since it can be worked and woven like animal and vegetable fibres into textiles. It has however, only during this century, become a significant component of the industrial and domestic environment In the period 1910 to 1978 annual world production of fibrous silicates increased dramatically from 0.1 to 5.1 million tonnes.

Table 1 Types of Asbestos Minerals



Asbestos occurs in many different forms (see Table 1), although chrysotile fibre forms the basic material for almost all the manufacturing activities of the asbestos industry owing to its greater fibre length and flexibility. Chrysotile is used in a range of products chiefly; asbestos cement, asbestos organic resins and textiles (including paper and felt).

1.2.2 Physical and Chemical Properties of Asbestos

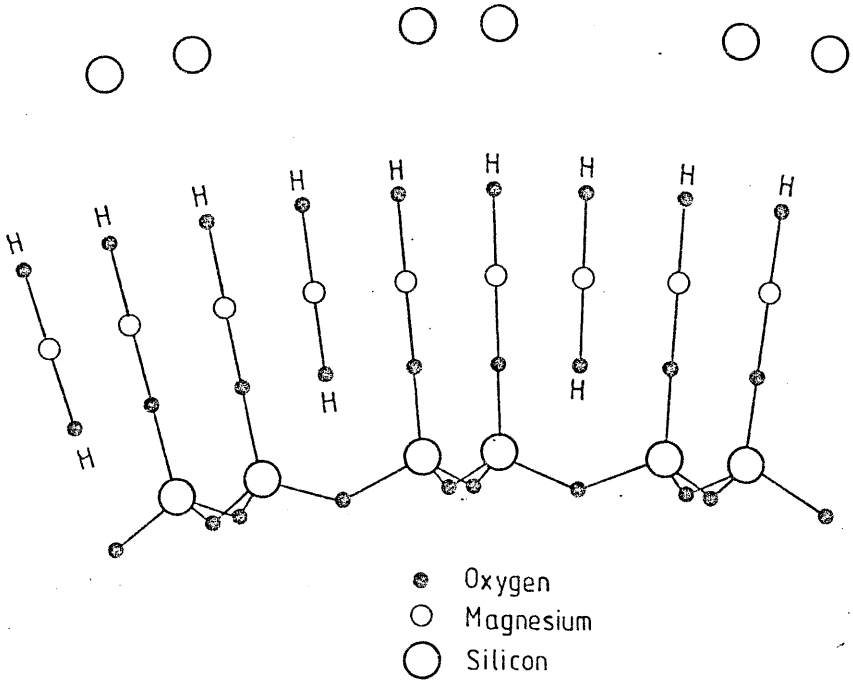
All forms of asbestos are metamorphic minerals that have developed from the parent, igneous rock through several stages of heating, cooling, pressure and hydrothermal action. They fall into two geological groups named serpentines and amphiboles and occur as cross-fibre seams of variable width (two to ten cm) in the host rock.

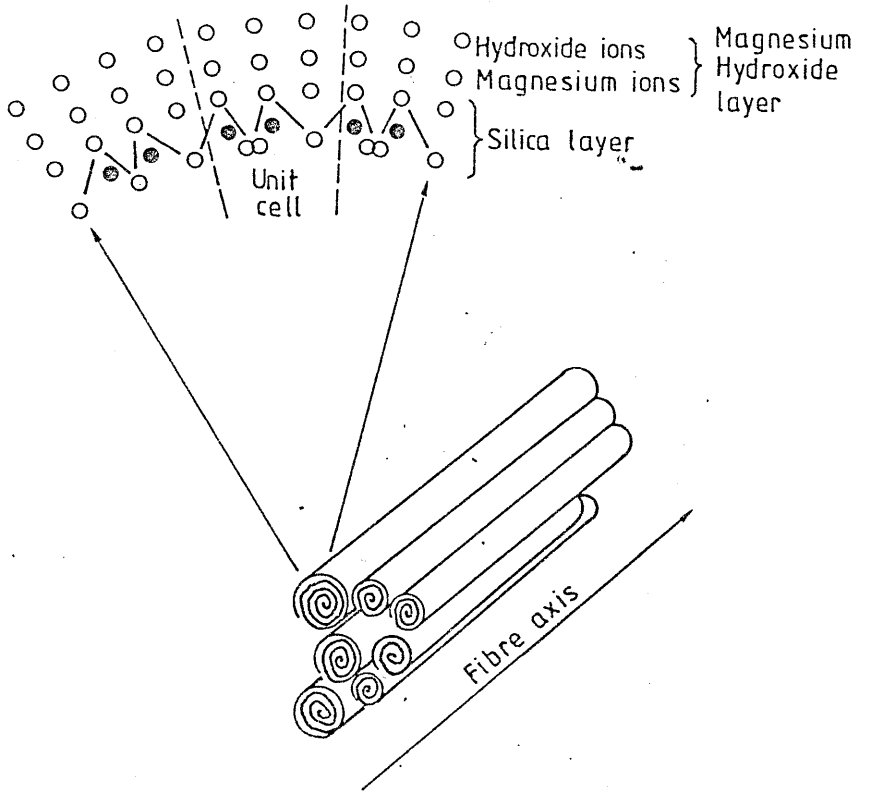
More than 90% of asbestos used in manufacture is the sheet silicate chrysotile, 'white asbestos', obtained mainly by open-cast mining in Canada, the Soviet Union and South Africa. The remainder, comprising the group of amphibole minerals, are chain silicates (see Table 1). The chemistry and physics of chrysotile are described in detail by Hodgson (1979). Two impurities found in the mineral are, carbon dioxide (linked with calcium and magnesium) and ferrous and ferric oxides arising from magnetite contamination.

1.2.3 Structure of Chrysotile Asbestos

A network of uniformly orientated molecules of silica tetrahedra form a linked sheet with a layer of magnesium hydroxide joined to one side of the net so that two out of

Figure 1 The structure of chrysotile asbestos (after Hodgson, 1966: 1979)





every three hydroxyl groups are replaced by oxygen at the apices of the tetrahedra. As a consequence, the silica matrix curves forming a scroll with the magnesium hydroxide on the side of greater curvature. This structure represents the ultimate fibril and has been shown to have an inner diameter of 11 nm and an outer diameter of 26nm. The inter-fibrillar space of the scroll is filled with silica gel (see Figure 1).

1.2.4 Physical Properties

Chrysotile has a highly hydroxylated, strongly polar surface. It therefore exerts a high surface potential in water, is hydrophilic and strongly basic. Suspensions of the fibre in water at 100°C yield solutions with a pH of 10 to 11. When titrated against 0.1 N hydrochloric acid the reaction proceeds in three stages:

1) Removal of about 5% of surface hydroxyl groups
2) Removal of 66% of total hydroxyl groups present, stripping of the surface layer of magnesium hydroxide and finally,
3) Completion of reaction to form hydrated silica.

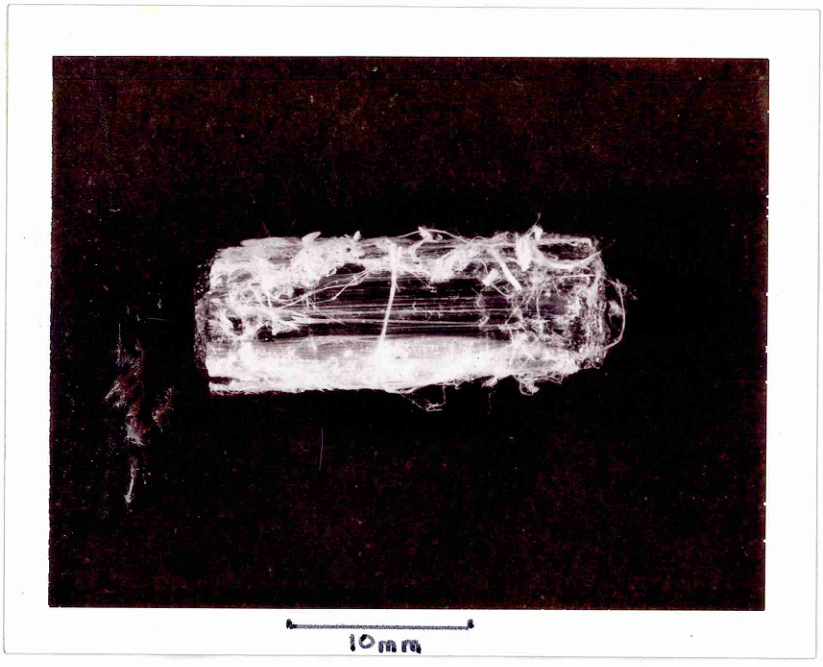
1.2.5 Surface Area of Chrysotile

The surface area of chrysotile preparations is dependent upon the extent to which the rock has been split into fibres during milling. Raw chrysotile, as shipped from the mine, has a surface area of 3 m^2/g (nitrogen method) and requires further fiberisation prior to processing. Typical asbestos used in manufacture has a surface area of 9 to 14 m^2/g.

Figure 2 The appearance of chrysotile

a) Rock fragment as shipped from mine

b) Fibre from dust magnified x 1000.



a

b

(nitrogen method) but the dust generated comprises still finer fibres (see Figure 2). It is this fine chrysotile dust that is the major hazard to workers in the asbestos industry. Thus, for the present study, the dust was freshly prepared by passing crushed pullings from chrysotile rock through a 100 mesh nylon sieve. The yield by this method consists of fibres 180 μm long or less with a surface area of 21.7 m^2/g. (krypton method) and approximates to that of inhaled industrial dust. The surface area measurements for this study were kindly carried out by Dr. J.A. Kitchener, Department of Mining and Mineral Technology, Royal School of Mines, London, S.W.7.

1.3.1 The Pathogenicity of Asbestos Dust

Any procedure involving the use of dry asbestos will generate free asbestos particles and is likely to release small fibres as dust into the atmosphere. It is in this form, 'dustry aerosols' that asbestos poses a hazard to health. Individuals inhaling the dust are at risk to fibrosis and neoplasia arising in the lungs and also the mesothelium lining the pleural and abdominal cavities. Calcification is a further possible complication especially in mesothelial lesions.

The first medically recorded case of asbestosis was reported by Murray in 1906 to the Departmental Committee for Compensation for Industrial Disease (HMSO, 1907). This patient was the last of eleven men who had died during employment in an asbestos spinning factory. In 1927 Cooke defined pulmonary asbestosis and in the same year McDonald

(1927) described the histology of the lesion. The following year Seiler (1928) also reported a case of pneumoconiosis caused by inhaling asbestos dust.

As a consequence, an investigation into the effects of asbestos dust on the lungs was initiated in 1928 by the Factory Department of the Home Office and the findings were reported in 1930 (Merewether and Price, 1930). The recommendations in this report formed the basis for the Asbestos Regulations (1931) which required the use of exhaust ventilation systems to prevent the escape of asbestos dust into workrooms and to provide breathing apparatus and protective clothing for use in areas where dust was unavoidable. The regulations, however, contained no requirement to perform dust particle counts on factory air samples. This form of control was not instituted until 1950 and a level of dust concentration was not set until 1960 when an upper limit of 177 asbestos particles/cc of air was advised by the Industrial Health Advisory Committee. Further regulations in 1969 widened the controls on the use of asbestos and set the upper limit of exposure at 2 fibres/cc. This limit was based on results from a study of 290 workers over periods of from 10 to 30 years. By 1980 most British factories had reduced chrysotile dust levels to 1 fibre/ml air and this is currently considered to be acceptable by the Health and Safety Commission. The statutory implementation of this limit is scheduled for 1st January 1983.

1.3.2 Asbestosis

Asbestosis is the term reserved to describe the condition of fibrosis of the lung parenchyma developing after inhalation of asbestos dust. It is generally accepted that, once initiated, chrysotile-induced fibrosis continues, notwithstanding removal from exposure, and eventually over a period of years obliterates much of the lung parechyma (Anderson, 1966; Zielhuis, 1977). However, not all individuals exposed to the dust develop asbestosis (Weiss, 1971) and the proportion of those who become affected is difficult to determine (Sluis-Cremer, 1970) principally because the degree of exposure cannot be assessed and the clinical course of the disease is protracted. In 1930, at the time of the Merewether and Price investigation, about 2,000 men and women were employed in the British asbestos industry. The findings were based on a sample of 363 of these exposed people and 95 cases of lung fibrosis were found. McVittie (1965), reporting on asbestosis in Great Britain, calculated that 0.5% of exposed individuals developed fibrosis. Beverley (1973), found 71 cases of asbestosis had been diagnosed in a total workforce of greater than 3,000 during a 66 year period in the asbestos plant (BBA Group Ltd.) on which this current study is based. Results from investigations on exposed shipyard workers showed an incidence of 0.6% in Britain (McMillan et al, 1978) and 1.1% in American shipyards (Murphy, 1978).

At present there is no clinical or laboratory investigation that can be used positively to incriminate asbestos as the specific agent responsible for subsequent pathology occurring after exposure. It is, therefore, difficult

to make a confident diagnosis in the early stage of the disease and it is only by inference that even a clearly established diffuse fibrosis of the lungs can be attributed to breathing the dust (Capel, 1979). To quote Buchanan (1979), 'this (diagnosis) is not as simple as it might appear, and especially in early cases, there is frequently disagreement even between experts'.

Routine screening of exposed factory personnel may reveal a suspicious lesion in a symptom-free individual. More commonly, the patient may complain of breathlessness, a non-productive cough and chest pains; the latter, on rare occasions, caused by a pneumothorax (air leaking into the pleural space). Diagnosis of asbestosis is based on:

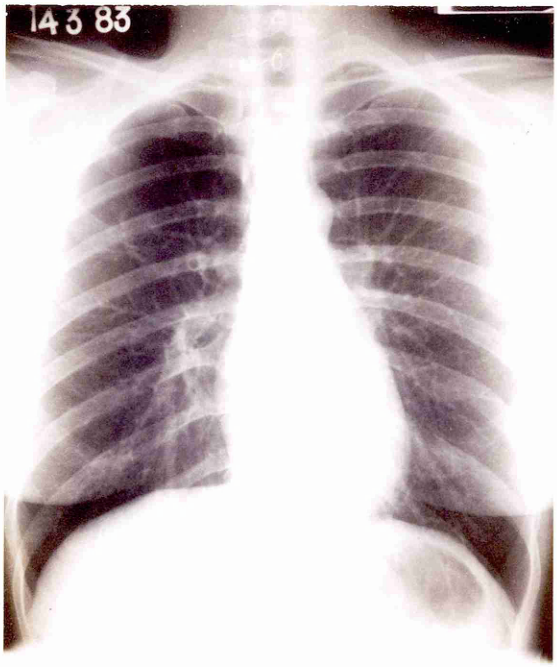
1) Careful history taking
2) Physical examination
3) Chest X-ray
4) Lung function measurements
5) Bronchoscopy
6) Lung biopsy

1.3.2 1) History

Questions concerning work experience and the materials used usually elicit evidence of exposure. However, unless the patient has been engaged in specific manufacturing tasks with asbestos, the duration and level of exposure may be difficult to assess. Non-industrial exposure can occur through handling and machining asbestos materials in the home, and occasionally, through dwelling in an area polluted by excessive asbestos emission.

Figure 3 Chest radiographs showing a) Normal lung picture
b) Diffuse fibrosis and shaggy heart shadow of patient with asbestosis.

a



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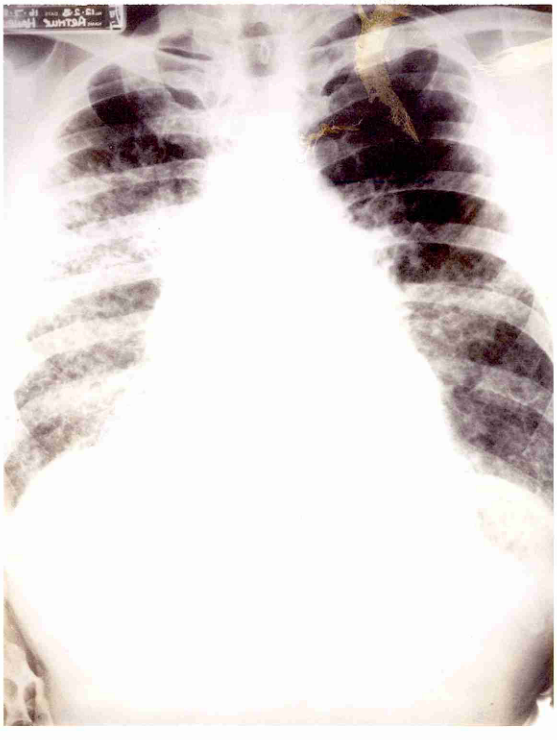


1.3.2 2) Physical Signs

Various combinations of these may be present. The patient's breathing may be seen to be laboured at rest. Percussion of the chest may reveal impaired resonance indicating thickening of the pleura, of the lungs and also pleural effusion. Finger clubbing and cyanosis may also be present. Natural free movement of air in the airways may be impaired in the smaller airways, especially the lower zones. This may be detected as crepitations, (fine crackles) that can be heard by means of a stethoscope. If the pleura is thickened sound transmission will be impaired.

1.3.2 3) Chest X-Ray

Evidence of early asbestosis is seen as a reticular fibrosis, usually present in the lower zones with extension into the periphery of linear shadows cast by the blood vessels. Subsequently, this develops into a diffuse fibrosis with shrinkage of the lungs and, occasionally, a shaggy heart shadow (see Figure 3). The extent of fibrosis is classified and graded according to international standards. Radiography will also display pleural thickening, plaquarding, calcification and neoplasia if these are present.

1.3.2 4) Lung Function Measurements

Lung function tests measure the mechanical efficiency of the lungs to breathe air and to accomplish the transfer of oxygen and carbon dioxide across the alveolar membrane. The physiological subdivisions of total lung capacity are shown in Figure 4. The principal measurements made are:

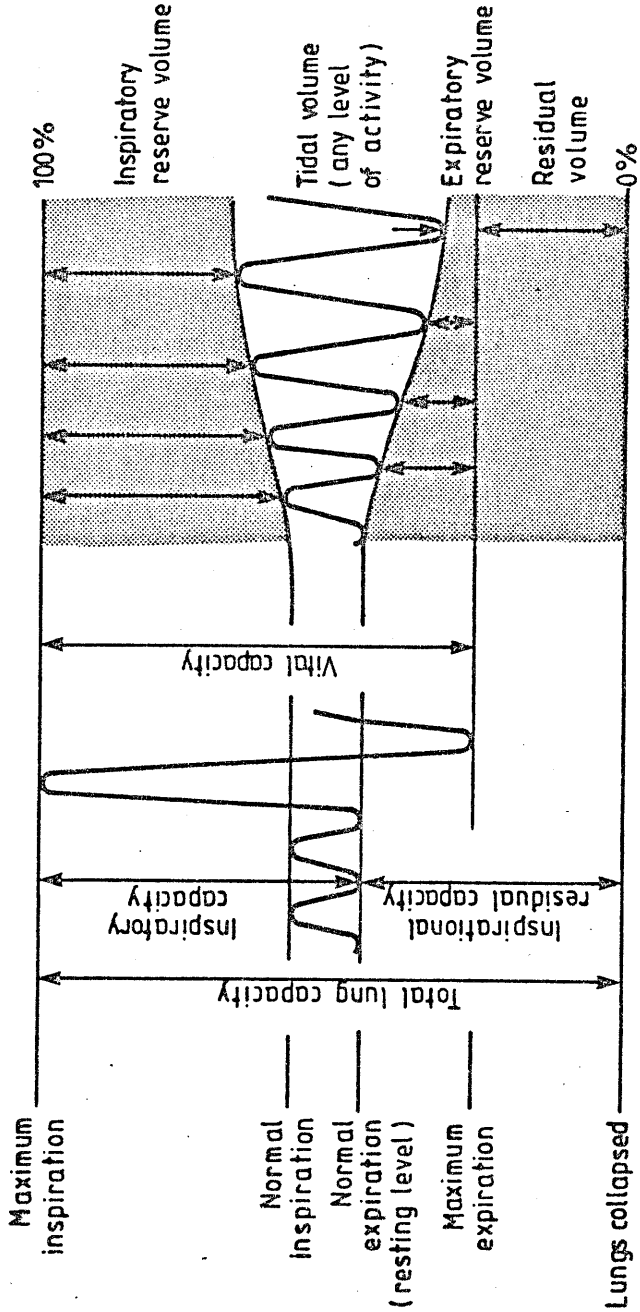


Figure 4 The physiological subdivisions of total lung capacity

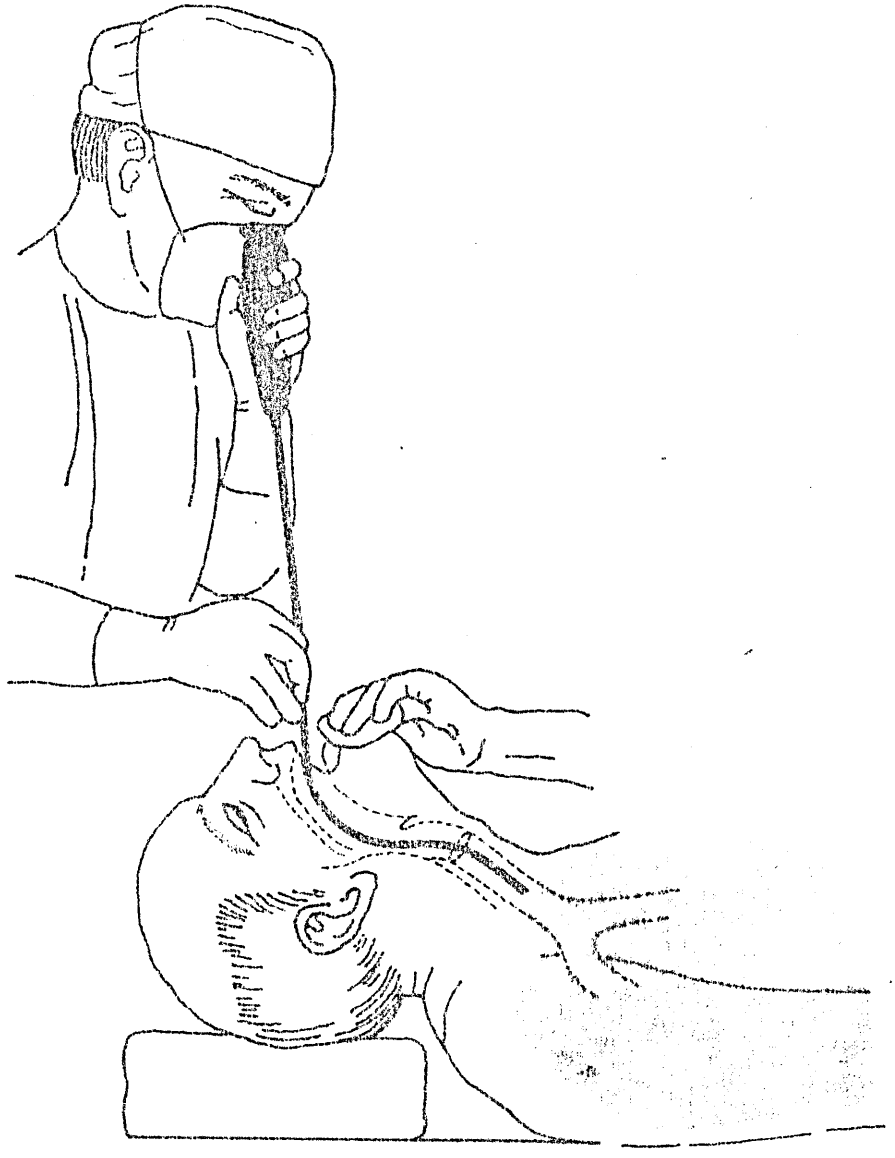
a) Peak expiratory flow rate (PEFR)	Detects major airway obstruction.
b) Forced vital capacity (FVC)	Detects defects in ventilation, and indicates possible causes.
c) Forced expiratory volume in 1 sec. (FEV_1)	
d) pCO_2	Assesses alveolar ventilation.
e) Transfer factor	Measures gas transfer function of the lungs.

These tests, and the techniques used for their estimation on BBA Group Ltd., personnel, are described by Hunt (1965). When serial investigations are carried out on individuals exposed to asbestos dust, retrospective studies show that abnormal progressive reduction in respiratory efficiency occurs in those exposed persons subsequently developing asbestosis four to five years before moderate radiological changes are seen (Hunt, 1965).

1.3.2 5) Bronchoscopy

The bronchoscope is a flexible canula at the tip of which is a light source. An image of the area illuminated immediately beyond the tip is carried by fibreoptics to an eyepiece held by the operator. The handpiece carries levers for manipulating the canula tip. The operation is performed on the fasting patient placed under light sedation. After inducing topical anaesthesia of the mouth, pharynx, tongue and larynx by spraying with 4% lignocaine the bronchoscope is introduced transorally as far as the larynx. Further anaesthesia is induced by injecting 5ml of 4% lignocaine through the aspiration duct of the bronchoscope before passing the tip past the vocal chords. See Figure 5. The tip of the

Figure 5 To show transoral insertion of the bronchoscope



canula is then directed into the bronchus leading to the lung segment chosen for study. A visual examination is made of the mucosa before proceeding to collect brushings, aspirate or biopsy specimens. During the procedure oxygen is administered if necessary via a nasal catheter.

Collection of Brush Specimens

A sheathed brush is passed along the canula until the tip is reached. The brush is unsheathed and swept over the mucosa. Immediately after resheathing the brush, it is withdrawn from the canula and the brush swept lightly over a microscope slide which is immediately immersed in fixature (usually 74 O.P. alcohol).

Collection of Washings

Material is aspirated from the airway by injecting 2 x 50ml of isotonic saline through the bronchoscope and immediately recovering the wash using the same syringe used for injecting the saline. The technique for processing the wash and the cytological findings are discussed in Chapter 3.

Collection of Biopsy Specimens

A biopsy forceps is introduced through the bronchoscope and directed toward the chosen site on the mucosa. The opened forceps is pushed firmly against the tissue surface and quickly closed. At this point the forceps is withdrawn from the bronchoscope and the biopsy fragment transferred

immediately into fixative (usually formal saline).

1.3.2 6) Needle Biopsy

Lung tissue from lesions in the peripheral lung fields can also be collected using a needle biopsy technique. The method is not used for less accessible lesions because of the risk of damage to major blood vessels.

With the patient placed under light sedation the skin overlying the chosen lung field is anaethesised and the needle introduced into the lung parenchyma. The operation is accomplished with the aid of intermittent exposure of the thoracic area to X-ray and the image displayed to the operator on a scanning screen. According to the type of needle used, a small tissue sample or aspirated cell sample is withdrawn and processed as for the bronchoscope specimens.

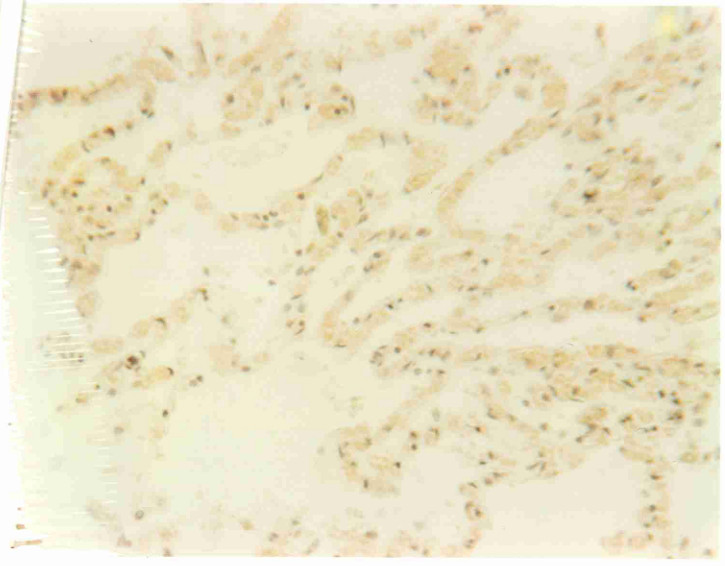
1.3.2 7) Histological Appearance of the Biopsy Section

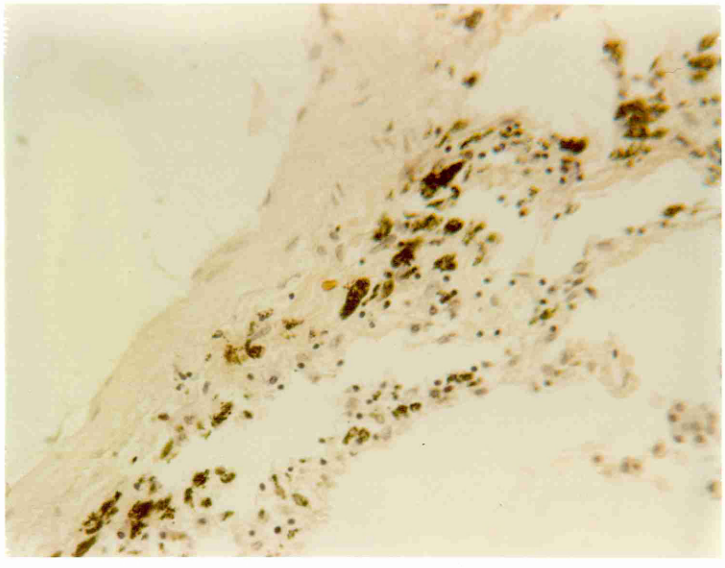
Parenchymal asbestosis is usually seen histologically as a diffuse fibrosis that cannot be distinguished from that induced by other causes. Initially, however, fibrosis affects the lower zones. This is because the weight of the lung causes the upper parts to be kept under greater stretch than the lower parts. During inspiration, air tends to be drawn into the lower zones resulting in the accumulation of retained fibre in these lung fields.

The histological diagnosis is based on inference if a diffuse interstitial fibrosis is found in the presence of asbestos as asbestos bodies and free fibre. The asbestos is usually seen in association with extracellular

Figure 1 Appearance of histological sections of lung at x 600 magnification.
Haematoxylin and eosin stain a) Normal lung, b) Asbestotic lung.

a





accumulations of carbon. See Figure 6. Asbestos fibre can be detected by viewing the section through cross Nicol prisms when its refringent property enables the fibre to be seen brightly illuminated against the otherwise dark field. Asbestos bodies and free asbestos fibre can also be demonstrated in sputum and bronchial washings although the finding serves only to confirm evidence of exposure.

1.4.1 The Bronchial Tree and Airway Mucosa

After the bifurcation of the trachea, each bronchus undergoes successive branching with progressive narrowing (see Figure 7), until, at the twelfth division, the average diameter of a bronchiole is about 2mm; this marks the beginning of the distal airway (Annot. Lancet, 1973). Here the lumina of the non-cartilagenous terminal bronchioles narrow to about 0.5mm. and form respiratory bronchioles. From each respiratory bronchiole radiate between two and eleven alveolar ducts; these end in vestibules called alveolar sacs formed from clusters of alveoli.

The upper airway mucosa comprises pseudostratified ciliated columnar epithelium blanketed with a mucoserous secretion produced by the submucosal glands (Lamb, 1968). Although the precise nature of the secretion is not yet established, Bowes and Corrin (1977) found that lysozyme is a major constituent. See Figure 8.

At the terminal bronchiole the epithelium is simple, columnar comprising ciliated and nonciliated (Clara) cells. The nonciliated cells have microvilli on the free surface. Both cell types contain secretory granules that may produce surfactant (Matthew and Martin, 1971). Chamberlain et al

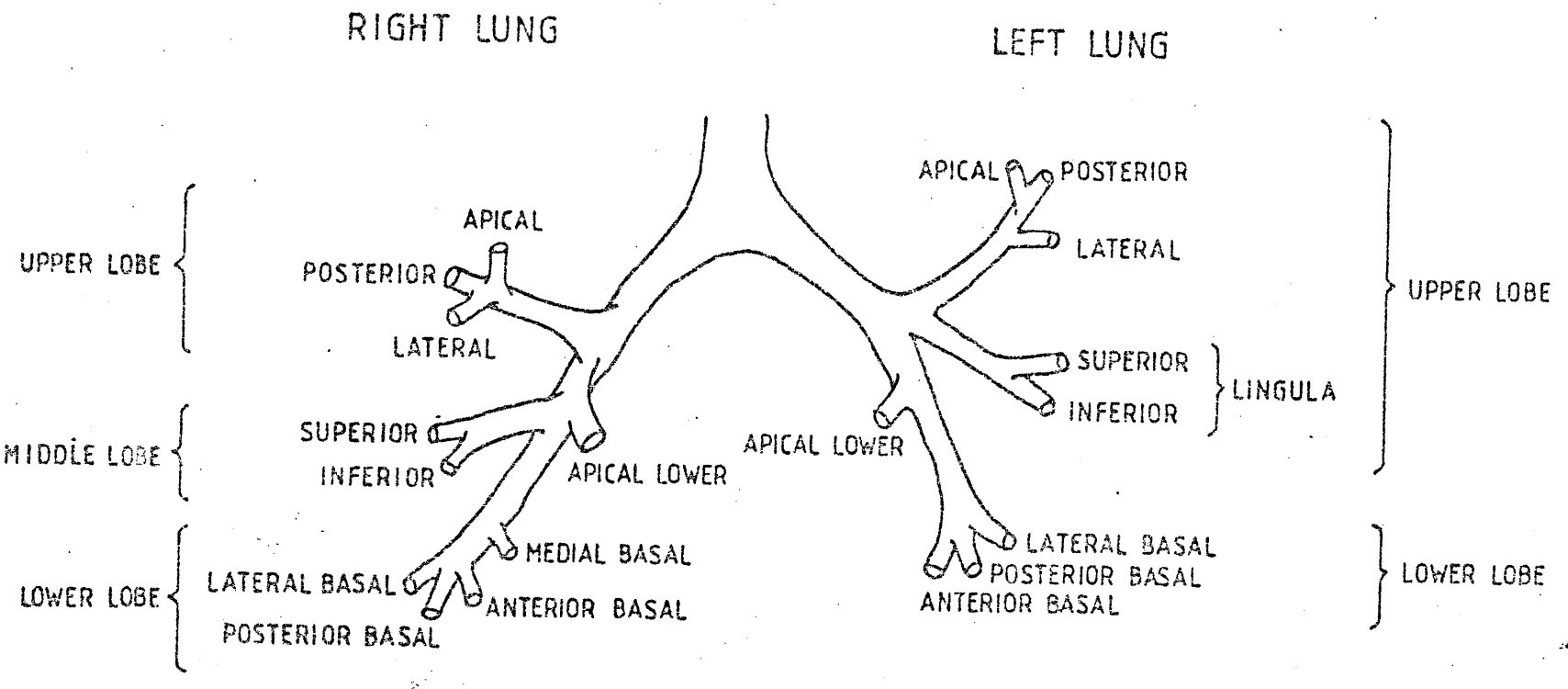


Figure 7 The major airways and lung segments (after Brewis, 1975)

(1973) confirmed the presence of the two cell types and noted that there are fewer nonciliated than ciliated cells. As the diameter of the terminal bronchiole narrows to 0.5mm it leads into the respiratory bronchiole. This is described by Maximow and Bloom (1948) as being 'relatively short, lined initially with ciliated epithelium that changes to low cuboidal cells ... a few alveoli bud off from the side opposite that along which runs the pulmonary artery'. Matthews and Martin (1971), however, describe this epithelium as cuboidal with microvilli projecting into the airway and noted that, whilst the cell appears to be equipped for secretion, large numbers of pinocytic vesicles are also present.

The alveolar ducts radiating from the respiratory bronchiole are lined with simple squamous epithelium that continues into the alveolar sac. At the alveolus this cell, known as a Type 1 (membranous) pneumocyte is interspersed with an occasional cuboidal cell, known as a Type 2 (granular) pneumocyte According to Desai et al (1978), this cell is the site of surfactant synthesis. The flat Type 1 pneumocyte is the predominant alveolar epithelial cell and its cytoplasm is intimately joined to its vicinal cells by impervious tight junctions. The coherent area of cytoplasm thus formed is the gas transfer area while the occasional Type 2 cell, usually found at the angles of the alveolus, secretes the lining of surfactant. Alveolar epithelium is relatively stable; the turnover time of the Type 1 cell is four to six weeks (Bowden, 1981). Cell renewal is accomplished by a two-stage process. The Type 1 cell is a committed, non-replicating cell and its replacement is achieved by the Type 2 pneumocyte which

has the dual role of surfactant secretor and reserve cell. The Type 1 cell is more susceptible to injury than the Type 2 cell whose progeny subsequently transform to Type 1 cells. When the Type 2 cell is in the reparative phase, the total Type 1 population can be replicated in three days.

It has been shown that alveolar development in embryo involves the primitive cuboidal epithelium invading the mesenchyme to form the bronchial tree and the terminal air sacs. After the formation of the air sacs the lining cells progressively differentiate to form Type 2 cells. As term approaches, the majority of the Type 2 cells further differentiate into Type 1 cells which overlie the capillaries.

1.5.1 Pulmonary Clearance of Respired Particles

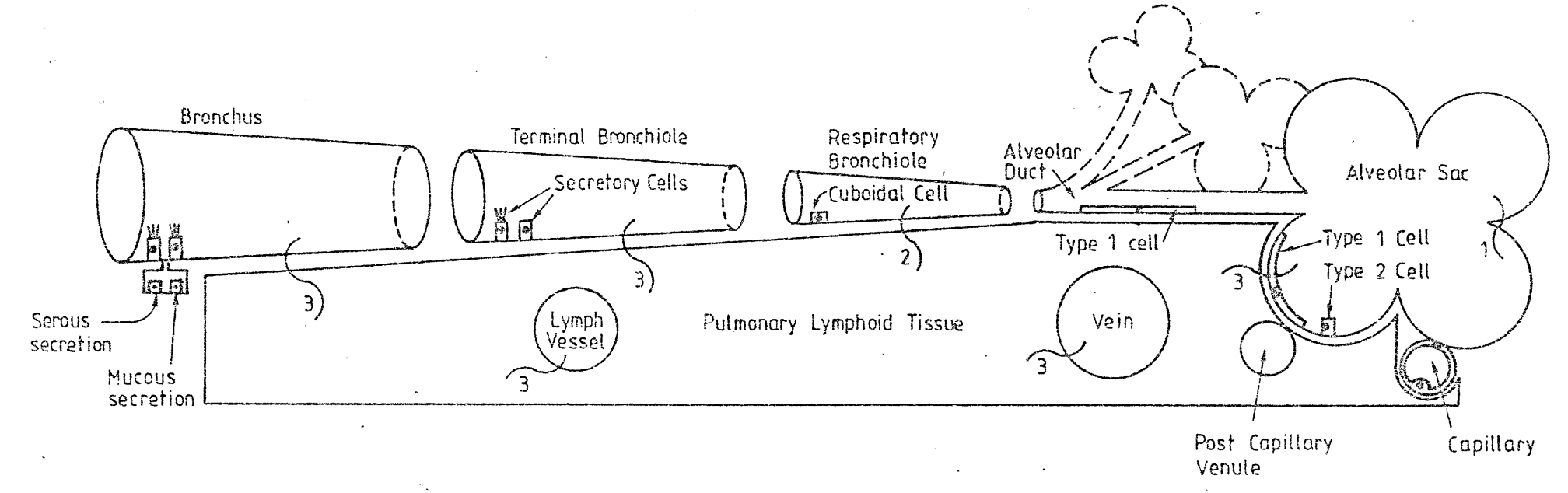
Man breathes about 10,000 litres of air in 24 hours so that inhaling even relatively low density particulate aerosols could result in the retention of a considerable weight of solid matter. Most particles that pass the naso-pharyngeal filter however, are subsequently expelled from the airway by means of the combined action of the ciliary bronchial mucosa and the cough reflex.

Inhaled particles travel a tortuous route to arrive at the broncho-alveolar region and it is probable that the size of the particle primarily determines the fate of a chrysotile fibre in the airway. Kimura (1970) showed that retention of polystyrene-latex spheres in the human lung is idiosyncratic and is only partly dependent on particle size and the respiratory tidal volume. Kimura's results showed that about 50% of spheres approximately 2 micron diameter and 30% of

those about 0.1 micron diameter are retained. This confirms the expectation that larger spheres are least likely to negotiate the airways unhindered. Harris and Fraser (1976) analysed possible deposition patterns of fibres in the respiratory system using a mathematical model of the human airway previously constructed by Weibel (1963). The predictions have, unfortunately, not been tested but they forecast that short, thin cylinders 25 x 1 micron diameter, have the greatest chance of reaching the distal airway and that small numbers of cylinders 200 x 1 to 4 micron diameter might also evade the naso-pharyngeal filter. Myrvik (1973) found that particles greater than 10 micron are usually trapped on the mucus lining the epithelium of the upper airway and are cleared almost exclusively by ciliary transport. Smaller particles can reach the distal airway and, for particles of 1 micron size, 50% may achieve the journey. Ashcroft and Heppleston (1973) reported that most of the retained fibre in the lungs of patients with asbestosis is of ultramicroscopic size and constitutes between 70 and 80% of the total retained fibre. Industrial dust contains fibres ranging in size from submicroscopic up to 180 microns in length and all sizes can be found in asbestotic lungs.

It is not known how translocation of retained asbestos particles occurs from the bronchiolar/alveolar mucosa to the lung parenchyma: it may be either mechanical or cell-assisted. During respiration, distension of the bronchial tree takes place as far as the terminal bronchioles with each inspiration so that the site of deposition of a chrysotile fibre need not

Figure 8 Inter-Relationships at the Distal Airway



Established macrophage (alveolar histiocyte) transmigration routes

1. Tubbs, R.R., Benjamin, S.P. Osborne, D.G. and Barenberg, S. (1978).
2. Matthews, J.L., and Martin, J.H. (1971).
3. Chamberlain D.W., Nopajaroonsri, C. and Simon, G.T. (1973).

be its final location on the mucosa. Gross et al (1973) have suggested that macrophages play no part in the penetration and lymphatic transport of particles. The alveolus is generally regarded as a passive structure during respiration thereby reducing the chance that a fibre would be suitably orientated to pierce the alveolar membrane and so pass into the parenchyma. During coughing, however, partial closure of the glottis produces impaction of particles in the alveolar ducts and this forces the contents into the alveolar sacs. This would enable some fibres to penetrate into the lung parenchyma. A mechanical mode of entry becomes more plausible when the 'contractile interstitial cells' of Kapanci et al (1974) are considered. These authors reported that 50% of alveolar interstitial cells contain contractile fibrils and they postulate that this endows the alveolus with an active role in respiration. It is generally accepted, however, that particle translocation is cell-assisted, Heppleston (1963(a)) considered that the primary entry of particles is via endocytosis by Type 2 pneumocytes that subsequently disintegrate. The spilled contents are then taken up by histiocytes; although in the same year Heppleston(b) also reported that 'migration of detached alveolar cells is a possible mechanism.'

It is unlikely that the accumulation of carbon in the lung parenchyma and hilar lymph nodes is primarily by mechanical penetration of the alveolar wall or by the weakly phagocytic Type 2 pneumocyte. Alveolar histiocytes are ubiquitous in lung tissue and histiocyte transmigration has been established between most lung tissues (see Figure 8). Also, large-scale recruitment of histiocytes is seen in lungs that have been exposed to air containing dust (Ferrin et al, 1965).

1.6.1 Aims of the Research Study

It is generally accepted that chrysotile can cause either or both fibrosis and neoplasia but the response by humans to insult by asbestos is idiosyncratic. The current study is concerned with asbestosis, a term reserved solely for irreversible lung fibrosis induced in some individuals after inhaling asbestos dust. Laboratory research into asbestosis has focussed principally on three areas:

1. The physical distribution and deposition of inhaled asbestos fibre and the morphological characteristics of retained chrysotile as found in the lungs post mortem.
2. The pathogenicity of asbestos with respect to particle size.
3. The dynamics of tissue damage including collagen formation, as found in experimental animals subjected to dust inhalation and extrapolated to the appearances found in human lungs post mortem.

The interaction between retained chrysotile fibre and secretion at the air/mucus interface of the airway mucosa constitutes a gap in published research. The lack of research study in this area is surprising in the light of two observations. First, retrospective studies have shown that impaired respiratory efficiency occurs in asbestosis sufferers four to five years before fibrosis becomes radiologically apparent (Hunt, 1965). This suggests that there is a protracted period

before the onset of irreversible fibrosis during which evidence of cell pathology may be detectable in the airway mucosa and its secretions. A second key point is that the cytotoxic property of chrysotile, first noticedby House (1964), is lost when the fibre is coated with various proteins such as haemoglobin (MacNab and Harington, 1967), saline dilutions of serum and bronchial secretion (Allinson, 1970), erythrocyte cell membranes (Schnitzer and Pundsack, 1970) and saline dilutions of foetal bovine serum and ox pulmonary surfactant (Desai and Hext, 1975). At the commencement of this study it was noticed that sputum and sections of lung from individuals exposed to chrysotile dust contained intact histiocytes with endocytosed carbon and chrysotile in the same cell and other histiocytes with endocytosed carbon and asbestos bodies in the same cell. Thus it can be inferred that lung histiocytes are able to endocytose chrysotile without incurring cytolysis.

All retained fibre must pass through extracellular secretion before it can cross the epithelial/endothelial cell barrier and it is evident that chrysotile fibre will adsorb protein in the process. It is of interest to discover what types of protein might be adsorbed and whether this could condition the cell response. This information might be obtained by analysis of sputum proteins and comparing the results from sputum donors classed according to status with respect to chrysotile exposure:

1. Donors unexposed to chrysotile.
2. Donors exposed to chrysotile without asbestosis.
3. Donors exposed to chrysotile with asbestosis.

The aims of this study were therefore to investigate the effect of inhaled chrysotile on the human respiratory tract mucosa and to examine whether there are differences between those who do and those who do not develop asbestosis. This was done by:

1. Analysing the extracellular proteins in sputa from the three donor groups, (Chapter 2).
2. Analysing the cell types present in sputa from the three donor groups, (Chapter 3).
3. Replicating in vitro the natural history of chrysotile fibre during its passage from the nares to the lung acini, (Chapters 4 and 5), and
4. Investigating the effect on human somatic cells of chrysotile treated as shown from 3. (Chapter 6).

The programme of research presented in this thesis therefore investigates, in logical progression, the series of events probably occurring as chrysotile fibres are carried along the human airway to the point where they become 'retained fibre' in an attempt to answer the following questions:

1. Does exposure to chrysotile bring about detectable changes in the proteins of the airway secretion?
2. Does inhaled chrysotile cause characteristic exfoliation from the airway mucosa?
3. Is there a general pattern of protein uptake onto inhaled chrysotile fibre from airway secretion?
4. What effect does chrysotile so coated have on the human lung histiocyte?

CHAPTER TWO

The Bronchoalveolar Secretion as Represented By Sputum

PART ONE

The Extracellular Proteins in Sputum

The Bronchoalveolar Secretion as Represented by Sputum

2.1.1 Introduction

In this section the extracellular proteins found in sputum from individuals employed in an asbestos processing factory (BBA Group Ltd.) are investigated and compared according to donor category (see Section 2.1.2).

Despite the importance of the bronchoalveolar secretion to the physiology and pathology of the respiratory system, little is known about the composition and structure of its components. It is difficult to collect normal bronchoalveolar secretion since all of the collecting procedures provoke hypersecretion. However, estimates have been made that the volume of secretion produced by the airway mucosa in 24 hours may range from 50-150ml. in healthy individuals (Widdicombe, 1978) increasing to 600ml in disease (Kilburn, 1968).

Histological evidence shows that the secretions comprising sputum originate in the serous and mucous cells of the tracheobronchial submucosal glands and the secretory cells of the airway epithelium (Lamb and Reid, 1969; Jones, 1978). These secretions form a mucous colloid that is not homogenous. Studies of mucus shear rates and of ciliary action suggest that the cilia are beating in a largely aqueous sol phase whilst the upper mucous gel, the 'visco-elastic layer', serves to protect the sol phase and the tissue surface from dehydration (Kilburn, 1968; Blake, 1973). A precise histochemical analytical method for examining bronchoalveolar secretion has not yet been devised and it is doubtful whether this can be achieved using fixed tissue. Most of the solutions used in fixing and processing

tissues can either degrade or remove some of the components of the secretions particularly the carbohydrate moities attached to proteins. Also, the minute amounts of some protein fractions, for example, the immunoglobulins, require extremely sensitive reagents. A possible approach might be the use of specific immunofluorescent antisera on unfixed tissue (Masson, Heremans, Prignott and Wauters, 1966). Information on the glycoprotein components of the tracheobronchial secretion has accrued from several histochemical tests the most important being the periodic acid-Schiff (PAS) reaction alone and in association with alcian blue at two levels of acidity, pH 1.0 and pH 2.6. These techniques are described by Pearce (1972). Several papers collate past work and report on further study, mainly on non-human lung tissue (Thomas, 1972: Lorenz, Korst, Simpson and Musser, 1957: Spicer, Chakrin, Wardell and Kendrick, 1971: Bernstein, Young, Hahn and Kikkawa, 1969) and an appraisal of differences between the human tracheobronchial tree and that of laboratory animals has been made by Korhonen, Holpainen and Paavolainen, (1969). The findings on human lung by the latter workers are supported by McCarthy and Reid (1964) and Lamb and Reid (1970). The conclusion drawn from these reports is that bronchial mucoproteins comprise a mixture of neutral and acidic components. The acidic fraction is mainly sialic acid (sialomucin) of which only part is labile to sialidase and a sulphated fraction (sulphomucin). A layer of sialomucin has been demonstrated on the alveolar surface and it is suggested that this may be an important factor in the protection of alveolar cells from injury (Adamson and Bowden, 1971). The production of lysozyme in the serous cells of the human bronchial glands has been reported by Bowes and Corrin (1977).

2.1.2 The Classification of Sputa Used in This Study

Donors were categorised into two classes according to occupational history:

1. Unexposed to chrysotile dust. This constitutes the control group.
2. Exposed to chrysotile dust.

The concentration of chrysotile in the air breathed by the exposed group varied from less than 1 fibre/ml air to 50+ fibres/ml air the higher levels having occurred many years ago. Individuals in the exposed group were subclassified into those without asbestosis and those with asbestosis. All people classed as asbestotic had been assessed as such by the Pneumoconiosis Medical Panel (Sheffield).

The volume of sputum collected from each donor in this study ranged between 0.6 and 2.0 ml., most samples being about 1.0 ml. but precise data on sputum volumes was not recorded.

2.1.3 Analysis of Sputum Proteins

Existing techniques in protein chemistry prohibit the use of unaltered sputum because of its viscosity and non-homogeneity. Workers embarking on analysis of respiratory tract secretion have overcome these problems by various methods:

1. Proteolysis using pepsin (Bukantz and Borns, 1958), trypsin (Biserte, Havez and Culvier, 1963), papain and pronase (Degand, Roussel and Lamblin, 1974).
2. Dialysis with water (Havez, Roussel, Degand and Biserte, 1967).

3. Mucus precipitation with Cetavlon (Atassi, Barker and Stacey, 1962).
4. Ultracentrifugation (Ryley and Brogan, 1968; Brogan, Ripley, Allen and Hutt, 1971).

2.1.4 Method of Preparing Sputum Used in This Study

All the above methods involve fractionation of the sputum proteins and this was deemed to be undesirable in the current study. It was found, during experiments using ultrasonic force for cell disintegration, that the viscosity of sputum was lost at a recognisable transition point (see below).

Although this was an independent finding, a later search of the literature yielded a similar report by Brogan (1960). The apparatus used in the current study was manufactured by the Schuco Division of Ultratec (America Caduceus Industries, New York). Two solid probes were used, one was a 4mm diameter titanium rod while the other was made from stainless steel and was 13mm. diameter. The work input of the unit was 125 watts. For sputa of volume less than 1 ml. the titanium probe was used and the stainless steel probe was employed for larger volumes. In either case the probe was positioned in the sputum at the point of maximum turbulence and left until the transition point was reached. This was seen as a sudden change to homogeneity in the sputum and was accompanied by a characteristic change in pitch of the sound emitted by the probe. Cells were subsequently removed from the sputum by centrifuging at 1,500g for 5 minutes. Further centrifuging at 14,000g for six minutes removed an

amorphous, opaque white fraction that stained with haematoxylin, Feulgen's reagent and the pyronin component of Unna-Pappenheim's stain indicating that it probably comprised cell debris.

For the purpose of the present study a range of analytical techniques was used to detect any differences between sputa from the three donor groups a) Unexposed to asbestos, b) Exposed to asbestos without asbestosis, and c) Exposed to asbestos with asbestosis. The scheme of the original approach - total protein assay, electrophoretic separation and immunoprecipitation - was subjected to considerable modification as the work progressed. The altered approach involved changes in immunoanalytical methods and the introduction of ion exchange analysis.

A preliminary investigation was carried out to determine whether exposure to ultrasonic force affected the behaviour of proteins. It was found that parallel analysis of ultrasonically treated and untreated serum from the same specimen yielded the same results.

2.2.1 Total Protein in Sputum

2.2.1 Materials and Method

Sputum was prepared as described in Section 2.1.4.

After investigating several chemical methods the technique adopted for the current study was developed from Wu's (1922) method for plasma proteins as follows:

Sputum solution	0.1ml
Deionised water	2.0ml
Folin and Ciocalteu reagent	0.1ml
Sodium carbonate 20% aqueous	2.0ml

The tubes were mixed by inversion, incubated 1 hour at 37°C, and read in an Eel colorimeter using Ilford filter 607 (band pass 578 - 720nm). The Folin and Ciocalteu reagent reacts with the phenyl groups in the tyrosine component of proteins, the concentration of which is 4.7g/100g in albumin and 6.7g/100g in globulin (Brand, Kassell and Saidel, 1944). Since electrophoresis revealed only small amounts of albumin in sputum compared with globulin (see later), human serum was used as the standard protein solution.

2.2.2 Results

The spread of total protein results from the three classes covered differing ranges:

Unexposed	790mg.
Exposed without asbestosis	862mg
Exposed with asbestosis	1345mg.

The mean results were: unexposed 587mg%, exposed without asbestosis 388mg%, and exposed with asbestosis 694mg%. These findings together with the standard deviation of the means are given in Table 2 which also gives the analysis of comparison of the means and shows that the mean result for the exposed without asbestosis class is significantly ($p = 0.05$) lower than the mean result for the other two classes.

2.2.3 Discussion

A search of the literature yielded few reports on total protein analysis of sputum and none of these related to asbestosis. Ryley and Brogan (1968), and Brogan, Riley,

Table 2 The Results for Total Protein in Sputum (mg.%)

Donor Class	Unexposed	Exposed without asbestosis	Exposed with asbestosis
Number of donors	14	16	14
Range of values	230-1020	138-1000	190-1535
Mean	587	388	694
± s.d.	225	212	305

Comparison of the Means

	Unexposed/ exposed without asbestosis	Unexposed/ exposed with asbestosis	exposed without/exposed with asbestosis
$t_{(calc)}$	2.57	1.10	3.26
$t_{(table)}$	2.05	2.06	2.05

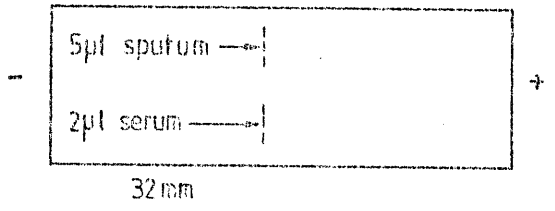
$p = 0.05$

Allen and Hutt (1971) investigated the sputum from patients with asthma, bronchitis and lung parenchymal damage and the results obtained ranged between 460 and 1145 mg.% protein. Their assays were made using a biuret method with albumin as the reference standard (Itzhaki and Gill, 1964). A mean value of 1000 mg.% total protein in bronchial secretions from nine laryngectomised patients was reported by Matthew, Spector, Lemm and Heremans (1966) and in another series of twelve similarly treated patients Kohler (1968) reports a mean value of 550 mg.% total protein; neither of these papers describes the method used for protein estimation.

In the current study, when the mean total protein concentration in sputa from the unexposed group is applied to Widdicombe's (1978) estimates of normal bronchial secretion, a mean daily output of 300 to 900 mg. protein is obtained for the lowest and highest estimates. It would seem, therefore, that maintenance of the respiratory tract mucosa, in healthy subjects, places a relatively small demand on dietary resource.

Statistical analysis of the mean total protein content of sputum in the three donor classes shows a significantly lower protein level in the 'exposed without asbestosis' group. ($p = 0.05$). Thus it can be inferred that dust inhalation induces hypersecretion which is achieved by the cells producing a diluted mucus with respect to protein content. It was thought that electrophoretic separation of sputum proteins might yield information on the causes of the low protein level in this group and possibly indicate abnormalities in individual electrophoretic fractions.

Figure 9 To show loading of protein solutions on electrophoresis strip



A.9

2.3.1 Electrophoresis of Sputum Proteins

2.3.1 Materials and Method

Sputum prepared as described in Section 2.1.4.

Standard normal serum.

Barbitone acetate buffer pH 8.6, ionic strength 0.05.

This solution is prepared as follows:

sodium acetate	6.50g.
barbitone soluble	8.87g.
barbitone	1.13g.
deionised water	2.0 l.

Vokam SAE 2761 D.C. power pack (Shandon Southern Products Ltd., Cheshire).

Phoro Slide cellulose acetate strips (Millipore (U.K.) Ltd., Harrow).

5% trichloracetic acid.

5 microlitres of sputum and 2 microlitres of serum were loaded onto a cellulose acetate strip 76mm x 25mm. See Figure 9 . The strips were then wetted with buffer and a current of 2.5 mA was passed for 15 minutes. Immediately after removal, the strips were fixed in 5% trichloracetic acid for two minutes and then stained for total protein or PAS reacting proteins using the Phoro Slide methods (Millipore, 1968) and allowed to dry. The resulting electrophoretograms were scanned by reflectance in a Chromoscan apparatus (Joyce Loebl Ltd., Newcastle) using a slit width 0.1mm, wedge 5-077D and filter appropriate to the staining method. Additional strips were stained for nucleic acid by Feulgen's method (Farrand, 1966) or orthotoluidine (Havez, Roussel, Degand and Biserte, 1967). Further electrophoretograms were

Figure 10 Chromoscan traces of electrophoresis strip stained for total proteins (Ponceau S stain)

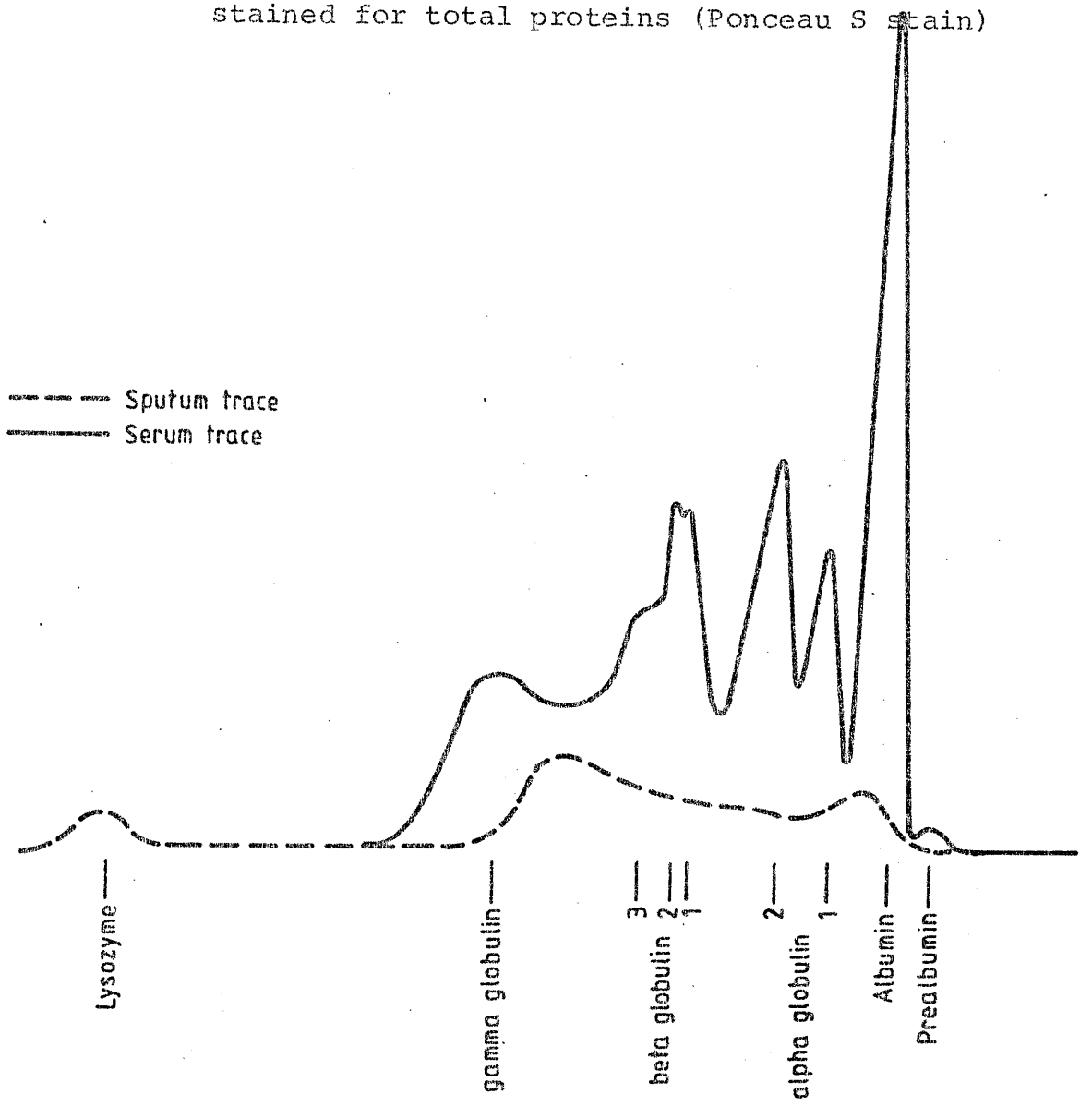
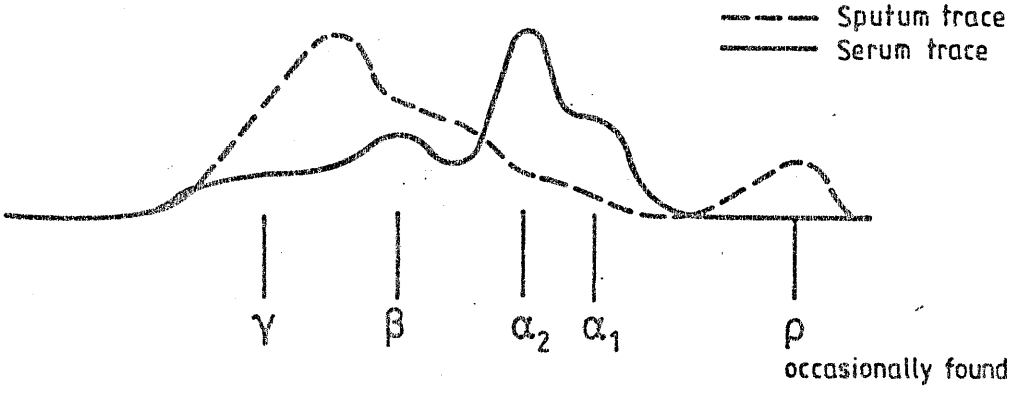


Figure 11 Chromoscan traces of electrophoresis strip stained for PAS reacting protein



stained for mucoprotein by the method of Lamb and Reid (1969) and also by the modification of the method by Butler (1973).

2.3.2 Results

The Chromoscan trace for total proteins is shown in Figure 10. It can be seen that the separation of serum proteins (solid line), is more pronounced than that of sputum (broken line). When the origin of the electrophoretogram was as shown in Figure 9, it was found that lysozyme had moved the same distance towards the cathode as had albumin towards the anode. By running the serum marker in parallel with sputum on the same strip the sputum alpha globulin in the trace shown in Figure 10 is located at the $alpha_2$ position. The sputum beta and gamma globulins are less distinctly identified and, in the figure, the peak can be seen between the $beta_3$ and gamma globulin positions. This pattern of beta and gamma globulin distribution was consistently found in the sputum electrophoretograms and, for this reason, it was decided to consider the sputum beta and gamma globulins as one fraction for the purposes of calculation.

Figure 11 shows the Chromoscan trace obtained when the strip was stained for PAS reacting proteins. Albumin and lysozyme are unstained by this method. The pattern for PAS positive protein shows increasing concentration from the alpha to the gamma locations. During this investigation an occasional specimen was found from all three donor classes that exhibited a fast-running PAS fraction at the albumin position but, since the fraction occurred in all three donor classes, it was not investigated further.

The strips stained for mucoproteins by the histological method of Lamb and Reid (1969) showed a uniform blue-stained area embracing the albumin and alpha globulin positions indicating acid glycoprotein while the beta/gamma area stained a mauve colour indicating predominantly neutral glycoprotein. Lysozyme was unstained by this method. Butler's (1973) modification of Lamb and Reid's (1969) staining sequence (mild hydrolysis with 0.05% sulphuric acid) caused no observable loss of acid glycoproteins.

The sputum separations on strips stained for nucleic acids were uniformly negative but a stained band was seen at the beta position in the serum separations.

Quantitative results were obtained from two series of electrophoretic analyses. The results from the first series are contained in Tables 3 and 4 . These show the percentage distribution of total protein and PAS staining protein after electrophoresis of several samples from each of the three donor classes. Albumin was not detected in two of the 13 samples in the 'exposed without asbestosis' class and alpha globulin was not found in three of the 13 'exposed without asbestosis' and one of the 11 'exposed with asbestosis' classes. Lysozyme was absent in 8 of the 23 sputa from the 'unexposed', 3 of the 13 sputa from the 'exposed without asbestosis' and 3 of the 11 sputa from the 'exposed with asbestosis' classes. From Table 5, it can be seen that the mean percentage concentration of albumin is significantly lower ($p = 0.05$) in the 'exposed without asbestosis' class than the other two classes and that the PAS staining beta and gamma fractions in this class are

Table 3 The Percentage Distribution of Total Protein After Electrophoresis

Donor class	Unexposed			Exposed without asbestosis			Exposed with asbestosis		
Number examined	23			13			11		
	Range	Mean	±s.d.	Range	Mean	±s.d.	Range	Mean	±s.d.
Albumin	12-30	19.8	5.7	0.27	15	3.8	12-30	19.7	5.1
alpha globulin	7-23	14.6	3.7	0-28	12.9	5.2	0-30	13.2	7.8
beta/gamma globulin	46-74	57.1	5.7	39-67	59.4	3.8	37-72	61.4	8.7
lysozyme	0-25	8.4	4.8	0-48	12.7	10.2	0-18	7.5	4.2

Table 4 The Percentage Distribution of PAS reacting Protein after Electrophoresis

Donor Class	Unexposed			Exposed without asbestosis			Exposed with asbestosis		
Number examined	23			13			11		
	Range	Mean	±s.d.	Range	Mean	±s.d.	Rang	Mean	±s.d.
alpha glycoprotein	16-61	37.7	11.3	29-61	43.5	9.5	14-64	41.4	12.2
beta glycoprotein	14-32	24.4	7.7	14-44	31.6	7.6	16-43	29.5	7.5
gamma glycoprotein	16-50	37.7	10.7	9-49	24.8	12.0	6-49	29.1	12.9

Table 5 Analysis of the Electrophoresis Results (Tables 3 and 4)

Comparison of the Means

	Exposed without/ exposed with asbestosis	Unexposed/ exposed without asbestosis	Unexposed/ exposed with asbestosis
albumin	2.53	2.80	0.10
alpha globulin	0.11	1.00	0.50
beta/gamma globulin	0.76	0.10	0.80
lysozyme	1.88	0.10	0.70
alpha glycoprotein	0.47	1.60	0.80
beta glycoprotein	0.22	2.70	1.80
gamma glycoprotein	0.84	3.10	1.80
$t_{(table)}$	2.08	2.04	2.04

$p = 0.05$

Table 6 The Distribution by Weight of the Sputum Protein Fractions After Electrophoresis

Total protein	455	500	550	620	800	940	1000	1300
albumin	118	140	143	87	176	133	260	220
alpha globulin	59	58	77	124	104	159	120	207
beta/gamma globulin	278	303	330	322	456	572	559	601
lysozyme	0	0	0	87	64	76	0	0
alpha glycoprotein	111	126	167	126	189	183	220	493
beta glycoprotein	101	59	86	124	217	183	192	137
gamma globulin	125	150	154	196	154	365	298	178

Unexposed donors (all values mg.%)

Total protein	500	575	625	650	2000
albumin	120	121	169	137	440
alpha globulin	70	92	88	78	140
beta/gamma globulin	310	357	369	300	1200
lysozyme	0	6	0	111	220
alpha glycoprotein	141	130	169	121	469
beta glycoprotein	114	157	64	136	308
gamma glycoprotein	125	162	224	121	563

without asbestosis

Total protein	525	550	600	800	825
albumin	115	116	120	128	173
alpha globulin	53	50	90	72	74
beta/gamma globulin	370	368	390	576	486
lysozyme	25	17	0	24	91
alpha glycoprotein	163	184	148	233	79
beta glycoprotein	96	138	96	175	241
gamma glycoprotein	163	96	235	240	241

with asbestosis

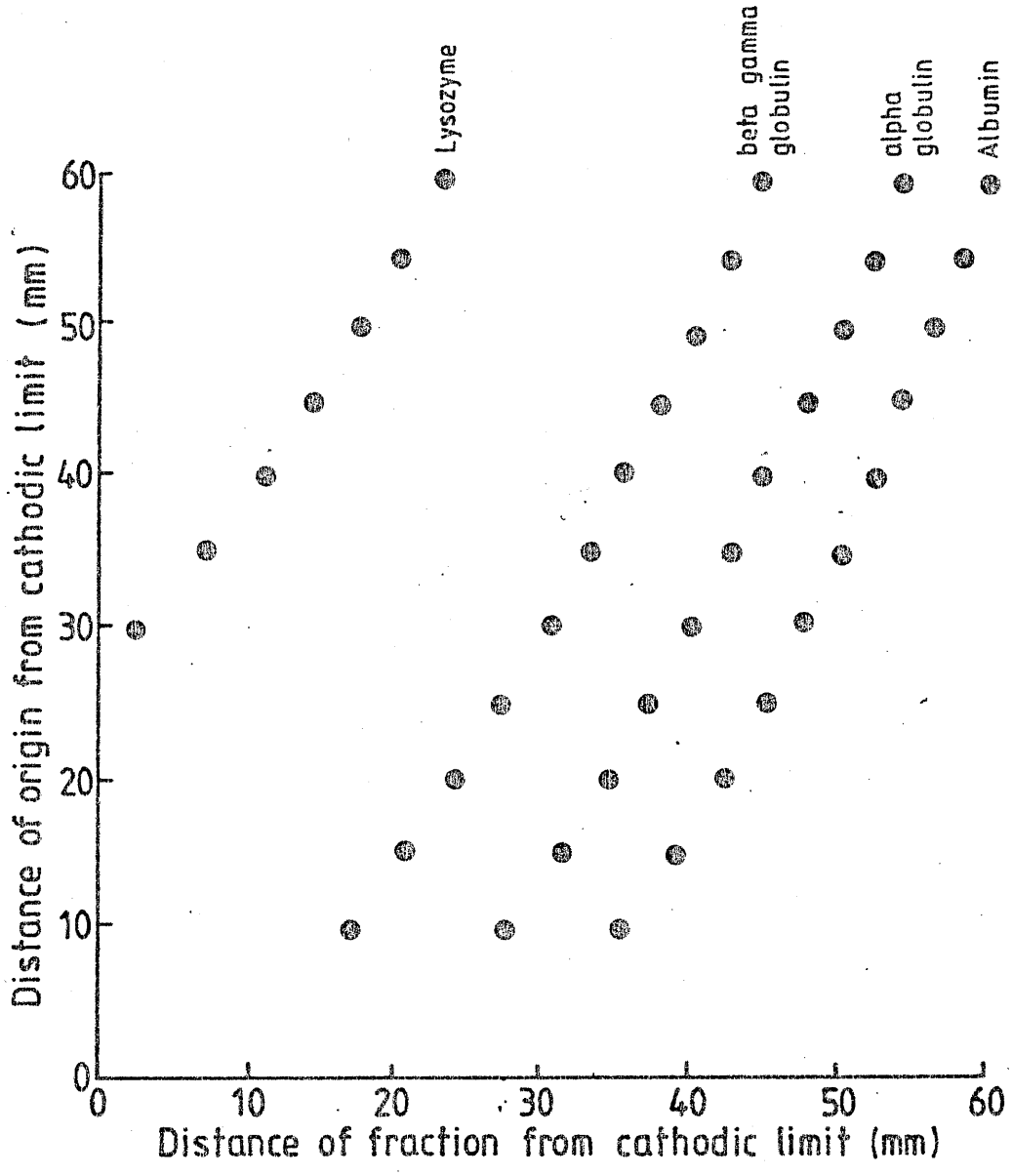
Exposed donors (all values mg.%)

Table 7 The Correlation Coefficients Between Total Protein and the Electrophoretic Components (w/w)

	Unexposed	Exposed without asbestosis	Exposed with asbestosis
No. examined	8	5	5
Albumin	0.721	0.993	0.795
alpha globuline	0.290	0.958	0.453
beta/gamma globulin	0.948	0.681	0.908
lysozyme	0.005	0.897	0.664
alpha glycoprotein	0.877	0.890	0.171
beta glycoprotein	0.597	0.918	0.876
gamma glycoprotein	0.492	0.977	0.730
$r_{(table)}$ =	0.666	0.811	0.811

$p = 0.05$

Figure 12 The electromobility of the sputum protein fractions



Total path length of potential gradient - 75 mm
Current : at start 10 mA 360 V
after 15 mins. 11.5 mA 285 V

reversed with respect to those of the other two classes.

A second series was made up from sputa selected from within the three classes to cover a range of total protein concentration to test correlation between the weight of total protein and the weight of individual fractions. The results are given in Table 6 in which it can be seen that lysozyme is not consistently detected in every sputum sample. Correlation is good ($p = 0.05$, Table 7), for all fractions except beta/gamma protein the the 'exposed without asbestosis' class whereas, in the 'unexposed' class correlation is found between total protein and albumin, beta/gamma globulin and PAS alpha glycoprotein. Sputa from the 'exposed with asbestosis' class show correlation between total protein, beta/gamma globulin and beta glycoprotein.

None of the protein fractions in the sputum electrophoretograms was electrophoretically immobile; it can be seen from Figure 12 that varying the location of the origin symmetrically displaced the fractions.

2.3.3 Discussion

Sputum from patients with respiratory disorders has been examined electrophoretically by several workers although preliminary partitioning of the sputum into sol and gel phases has usually been employed. Atassi, Barker, Houghton and Mullard (1961) and Laval, Meyer and Rosier (1961), working with whole sputum, found an electrophoretically immobile fraction. This observation was first reported by Warfvinge (1955). The immobile fraction was not found in the present study and neither was nucleic acid which has been noted by Havez, Roussel, Degand and Biserte (1967) to occur immediately in

front of the albumin position. The absence of these two components in the present study may be due to the fact that most of these substances were removed when the ultrasonically-treated sputa were centrifuged at 14,000g (see Section 2.1.4, Preparation of Sputum). This is a step not included by previous researchers. It is likely that both the immobile and the nucleic acid fractions found by those workers were due to cell debris and bacteria that are always present in sputum but were removed in the present work during centrifugation.

Brogan, Ryley, Allen and Hutt (1971) studied the sputum from patients with chronic bronchitis and found that lysozyme was consistently present. In the present study lysozyme was not found in sputum from 8 out of 23 individuals in the 'unexposed' class. This comparison is made since, in the absence of other lung pathology, sputum production is an accepted indicator of chronic bronchitis. The fact that lysozyme was not regularly found in the donor classes raises the possibility that particular proteins may be utilised or degraded in bronchial secretion. Lysozyme is a bacteriolytic agent and three sources contribute this substance to bronchial secretion; serum, phage cells (neutrophils and lung histiocytes) (Klockars and Reitamo, 1975), and the serous cells of the bronchial glands (Bowes and Corrin, 1977), the latter being the main supplier. In the series, lysozyme values ranged between 0 to 220 mg.% and the levels are unrelated to total protein in the 'unexposed' and 'exposed with asbestosis' groups. This suggests that, when none is found, it has been

utilised in controlling the bacterial burden carried by a chronically-infected airway mucosa. Another possible explanation for the absence of lysozyme in sputum, is that it has complexed with albumin. The binding of albumin with lysozyme was reported by Steiner (1953) and the electrophoresis results in the current study show that the density of surface charge on albumin (M.Wt. 66,300) and lysozyme (M.Wt. 15,000) is equal and opposite, See Figure 10.

From Tables 3 and 5 it can be seen that sputum from exposed persons without asbestosis contains proportionately less albumin than sputa obtained from the other two groups. Thus the significantly lower total protein found in the exposed without asbestosis group (Table 5) may be due wholly or in part to a reduced albumin content.

Table 5 shows significant differences ($p = 0.05$) in the percentage distribution of beta and gamma glycoproteins between the unexposed and the exposed without asbestosis classes. This difference is also shown in Table 7 where it can be seen that, whilst alpha, beta, and gamma glycoprotein correlate with total protein in the exposed group, only alpha glycoprotein correlates with total protein in the unexposed group. The table also shows that the total beta/gamma fraction of the exposed group does not correlate with total protein. This result would be expected if exposure induces a change in the balance of the secretion containing the non-glycoprotein fraction and if this change was reversed once fibrosis is established.

To study the beta/gamma fraction in more detail it was

decided to identify specific proteins in sputum utilising immunoprecipitation with as wide a range of specific antisera as possible but with particular reference to the gamma globulins.

2.4.1 3) Immunoanalysis

2.4.1.1 a) Immunodiffusion in buffered agar

2.4.1.2 Materials and method

Sputum prepared as described in Section 2.1.4.

Specific antisera (Hoechst (UK) Ltd., Hounslow)

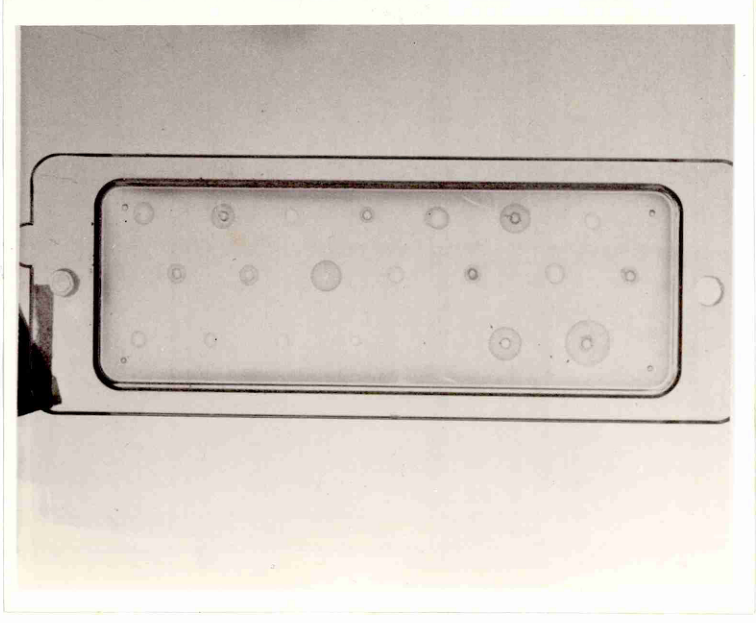
Buffered agar.

Buffered agar was prepared by dissolving 20g. 'immuno-diffusion quality' agar (B.D.H. Ltd., Poole) in a litre of buffer comprising:

Barbituric acid	1.66g.
Barbitone sodium	10.50g.
Calcium lactate	1.54g.
Deionised water	1 litre

The solution was dispensed in 10ml volumes into 1 oz. glass universal containers. For use, the agar was melted in a waterbath at 100°C after which the bottle was allowed to cool to 56°C. The chosen antiserum (0.1 ml) was drawn into a 1 ml. polystyrene syringe followed by 0.9 ml molten buffered agar. The plunger was further extended until an air bubble was drawn into the syringe. Agar and serum were then quickly mixed by inversion and expelled onto a Perspex tray 24mm x 75mm x 1 mm deep that was resting on a level plate. The tray was covered and allowed to cool. With the cover removed, the tray

Figure 13 Radial immunodiffusion in buffered agar (anti-albumin vs sputum)



was placed over a glass template marked in three rows of seven loci: each locus was 10mm from its neighbour (see Figure 13). A hole was punched in the agar over each location using a piece of 1mm diameter polythene tubing. Using a micropipette, 10 microlitres of sputum were added to each well and identified with reference to the grid. The cover was replaced and the tray sealed in a moist chamber at room temperature, 21°C. Immunoprecipitation was maximal after 3 days when surplus serum and sputum proteins were removed by washing in several changes of 5% sodium chloride for 4 hours. Immunoprecipitates were then stained with 0.2% Ponceau S in 5% trichloracetic acid for 5 minutes after which surplus stain was washed away with 5% acetic acid. Immunoprecipitates were seen as red annulae (see Figure 13).

2.4.1.3 Results

Sputum from 7 donors in the 'unexposed' class and 5 from the 'exposed with asbestosis' class were challenged with 15 specific antisera. A typical result with the technique is shown in Figure 13: this result was obtained after challenging sputa with anti-albumin serum. The figure is reproduced at the actual size and it can be seen that the method presents difficulties in measuring the diameter of immunoprecipitates especially when the concentration of albumin in the sputum is low. In addition, the 'target' character of the immunoprecipitate suggests that precipitation is not uniform; the ring at the sides of most wells is more darkly staining than the rest of the annular precipitate

indicating higher protein concentration at that location.

Of the 15 specific proteins sought only one, gamma a was found in all specimens (see Table 8), and the incidence of the remainder varied between 0 and 80%. With the exception of gamma g, a and m, the detection frequency % of proteins was higher in the asbestosis class than the unexposed class.

2.4.1.4 Discussion

Radial immunodiffusion was investigated as an alternative to immunoelectrophoresis because the latter method ('Immunophoroslide', Millipore (UK) Ltd., London), had proved capricious when applied to the detection of proteins in sputum. A disadvantage in this immunodiffusion method was the irregular distribution of the immunoprecipitate since this casts doubt on quantitative assessment of the protein.

The finding that gamma a was the only protein in the test group to be consistently demonstrated was not unexpected: it is the only protein of the group that is produced, in part, by secretory epithelium and Stockley and Burnett (1980) showed that the amount of locally produced gamma a does not change in 'active chest infection.' However, when the results in Table 8 are compared with the collated data from several sources, (Table 9), it can be seen that discrepancies occur. All previous authors listed in the table, consistently found albumin and gamma g in sputum in contrast with the current findings although the results for prealbumin, haptoglobulin, $beta_1$ $beta_1$A globulin, transferrin C and $alpha_1$ acid

Table 8 Proteins Detected in Sputum Using the Radial Immunodiffusion in Agar Method

Sputum donor status	Unexposed		Exposed with asbestosis	
Number examined	7		5	
	Incidence		Incidence	
	Number	%	Number	%
Prealbumin	1	14.3	2	40
Albumin	4	57.1	3	60
Haptoglobulin	0		3	60
Ceruloplasmin	2	28.5	4	80
Alpha macroglobulin	2	28.5	3	60
Beta, Cbeta, A globulin	0		1	20
Transferrin	7	100	3	60
gamma g	5	71.4	3	60
gamma a	7	100	5	100
gamma m	2	28.5	0	
$alpha_1$ acid glycoprotein	2	28.5	1	20
$alpha_2$ HS glycoprotein	3	42.8	2	40
$beta_2$ glycoprotein	2	28.5	4	80
$alpha_1$ lipoprotein	2	28.5	4	80
beta lipoprotein	1	14.3	4	80

Table 9 Collated Immunological Data for Proteins Found in Sputum (references below)

Anti serum against	Result		Reference
Whole human serum	+		1
Albumin	+		1,2,4,5,6,7;
gamma a	+		2,3,5,6,7
gamma g	+		2,5,6,7
$alpha_1$ antitrypsin	±		2,6,7
Haptoglobulin	±		2,5,6,7
alpha macroglobulin	±		2,5,7
Haemopexin	±		2,5,6,7
Transferrin	±	(+6)	2,3,5,6,7
$beta_1A$ $beta_1C$ globulin	±		2,5,6,7
$alpha_1$ acid glycoprotein	±	(+5 & 6)	2,5,6,7
$alpha_2$ antitrypsin	±		5
Prealbumin	±		7

+ = always found. ± = sometimes found. - = not found.

References:

1. Brogan T.D. (1960)

2. Schulz, A.L., Heremans, J.F. (1966).

3. Havez, R., Roussel, P., (1967)

4. Ryley, H.C., Brogan, T.D. (1968)

5. Kohler, H. (1968).

6. Ryley, H.C. (1970).

7. Brogan, T.D., Ryley, H.C., Allen, L., Hutt, H. (1971).

Table 10 Proteins detected in sputum using the radial immunodiffusion on cellulose acetate film method

Sputum donor status	Unexposed			Exposed without asbestosis			Exposed with asbestosis		
Number examined	25			22			22		
	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.
Whole serum	1.5-7.0	2.98	1.09	3.3-7.5	4.47	1.85	2.0-6.7	3.96	1.63
Prealbumin	0.0-3.0	1.24	1.29	0.0-3.0	0.85	1.16	0.0-3.2	0.72	1.11
Albumin	0.0-6.0	2.93	1.44	2.0-8.0	3.84	2.0	0.0-10.1	3.65	2.09
Haptoglobulin	0.0-6.5	2.34	1.35	2.0-5.9	3.03	1.63	0.0-6.5	2.63	1.22
Ceruloplasmin	0.0-5.0	1.78	1.0	0.0-2.5	0.85	1.13	0.0-3.2	1.11	1.16
$alpha_2$ macroglobulin	0.0-3.0	1.32	1.43	0.0-2.4	0.60	1.00	2.0-6.0	3.83	1.33
$beta_1C beta_1A$ globulin	0.0-4.5	2.06	0.77	0.0-2.1	1.10	1.03	0.0-2.8	1.00	1.06
gamma a	2.3-5.5	3.93	3.78	3.5-3.9	3.18	0.48	1.0-5.0	3.25	1.43
gamma g	2.0-5.0	2.84	0.82	0.0-7.5	3.89	1.83	1.0-8.2	3.45	1.35
gamma m	0.0-4.5	2.02	1.11	0.0-2.3	1.14	1.06	0.0-2.9	0.83	0.96
gamma e	0.0-6.5	2.17	1.65	0.0-3.0	1.25	1.78	0.0-2.2	0.56	0.86
$alpha_1$ acid glycoprotein	1.0-5.0	3.14	1.37	0.0-3.9	1.86	1.03	1.0-6.0	3.87	1.16
$alpha_2$HS glycoprotein	0.0-5.0	1.93	1.51	0.0-2.5	0.52	0.98	0.0-5.0	1.64	1.26
$beta_2$glycoprotein	0.0-5.0	2.16	1.14	0.0-3.0	0.93	1.12	0.0-2.0	0.64	0.79
$alpha_1$ lipoprotein	0.0-3.5	2.19	3.91	0.0-2.8	0.95	1.18	0.0-2.0	0.45	0.86
beta lipoprotein	0.0-4.5	2.48	3.23	0.0-2.7	0.55	0.97	0.0-1.5	0.38	0.66

Values are given as diameter of immunoprecipitate (mm)

Table 11 The Mean and Variance of the Diameters of Immunoprecipitation Obtained for Six Specific Protein Fractions

Donor Status	Unexposed		Exposed without asbestosis		Exposed with asbestosis	
	Mean	Variance	Mean	Variance	Mean	Variance
Albumin	2.93	1.98	0.84	2.00	3.65	4.19
Haptoglob-ulin	2.34	1.75	3.03	2.55	2.63	1.41
Transferrin	2.27	0.34	1.63	1.12	3.83	1.69
gamma a	3.93	13.74	3.18	0.22	3.25	1.95
gamma g	2.84	0.64	3.89	3.20	3.45	1.74
$alpha_1$ acid glycoprotein	3.14	1.79	1.86	1.01	3.87	1.29

Table 12 Analysis of the Diameters of the Immunoprecipitates of Six Specific Protein Fractions

Variance Ratio Test

	Exposed with/ exposed without asbestosis	Exposed without asbestosis/ unexposed	Exposed with asbestosis/ unexposed
Albumin	1.09	1.93	2.12*
Hapto-globulin	1.81	1.46	1.24
Transferrin	1.51	3.29*	4.97*
gamma a	4.06*	28.63*	7.05*
gamma g	1.05	2.86*	2.72*
$alpha_1$ acid glycoprotein	1.25	1.77	1.39
F=	2.0	2.0	2.0

p = 0.05

glycoprotein are similar in both tables. It should be noted, however, that in Table 3 'the percentage distribution of total protein after electrophoresis' the group of 13 'exposed without asbestosis' includes three sputa that contained no detectable albumin.

Because of the discrepancies and because of the relatively large volumes of sputum and antiserum required for the technique, it was decided to design an alternative immunodiffusion method.

2.4.2.1 3b) Immunodiffusion on cellulose acetate film

2.4.2.2 Materials and Method

Sputum prepared as described in Section 2.1.4.

Barbitone acetate buffer as for Electrophoresis, Section 2.3.1.

Specific antisera (Hoechst (UK) Hounslow).

Millipore cellulose acetate film ESWM 0440 R (Millipore (UK) Ltd., London)

The Millipore film was cut into rectangles 54mm x 108mm. These were then impregnated with a 1:10 dilution of the specific antiserum (0.08 ml antiserum in 0.72ml barbitone acetate buffer). A perforated reference grid was then placed over the film and a sample (0.5 microlitre) of each test sputum was added to the film through a perforation noting the grid reference for each test sputum. The film was then sealed between glass plates and incubated at 37^{o} for 18 hours. It was then washed free of unreacted protein with three consecutive washers in 5% sodium chloride at 37^{o}C. The film was stained with 0.2% Ponceau S in 5% trichloroacetic acid for

5 minutes. Surplus stain was removed by irrigation with deionised water. The film was then blotted and dried on a warm plate at 50°C for 5 minutes.

Immunoprecipitates appeared as red annulae.

2.4.2.3 Results

Sputa from the three donor classes were challenged with 16 specific antisera. Some samples were found in all donor classes that gave negative results for one or more of the following ten proteins:

Prealbumin

Ceruloplasmin

alpha macroglobulin

$beta_1$C$beta_1$A globulin

$alpha_2$ HS glycoprotein

$beta_2$ glycoprotein

$alpha_1$ lipoprotein

beta lipoprotein

gamma m

gamma e

The results are shown in Table 10 and an analysis of the six remaining proteins given in Tables 11 and 12 show that at $p = 0.05$, significant differences in albumin content were found between the unexposed and exposed with asbestosis but not the exposed without asbestosis classes. The mean transferrin level was lowest in the exposed without asbestosis class and highest in the exposed with asbestosis class. The mean level of gamma a was highest in the unexposed group and lowest in the exposed without asbestosis class, whilst

gamma g was lowest in the unexposed class and highest in the exposed without asbestosis class. Haptoglobulin and alpha$_1$ acid glycoprotein levels showed no significant difference between the mean diameters of the three groups.

2.4.2.4 Discussion

The identification of proteins by immunoprecipitation requires specific antisera that are costly to prepare. Commercially available products are virtually exclusively confined to specific antisera against known plasma protein fractions. Comparison between findings from this study and the results obtained by other workers, see Table 9, confirm the irregularity of detection in sputum, of most of the plasma components. It can be reasonably inferred, however, that plasma is a constituent of sputum if not of 'normal' bronchial secretion.

Sixteen antisera against plasma fractions were used in the current study and the results show that ten proteins (see list page 62) are eliminated as sputum protein markers for asbestos exposure because each of the ten may not be detected in particular sputa from all three donor classes. The immunodiffusion results also outrule two further fractions, haptoglobulin and alpha$_1$ acid glycoprotein, as asbestos markers because the mean diameters obtained for these two proteins show no significant difference between the donor classes.

The albumin results appear to be disappointing. In the absence of trauma, albumin is an accepted indicator of changes in membrane permeability. The electrophoresis findings

suggest that 1) the permeability of the airway mucosa is not changed during exposure, 2) hypersecretion accounts for the fall in albumin during exposure and 3) the stimulus to hypersecretion is removed or occluded when fibrosis is established. If the immunoprecipitate diameter is a true index of protein concentration, a significantly lower mean diameter should result for the 'exposed without asbestosis' class when it is compared with the other two classes. No significant difference was found between the diameters of the 'unexposed' and the 'exposed without asbestosis' groups and the mean diameter of the group with asbestosis was significantly greater than the exposed without asbestosis group. An explanation for this behaviour might be the complexing of albumin with lysozyme (see Section 2.3.3), the effect of which would be to retard the diffusion rate compared with albumin alone. In this case, the random variation in the lysozyme content of sputum (see Table 6) would further confuse the immunodiffusion results.

Transferrin is concerned with the binding and transportation of iron. The immunodiffusion results for this protein show significantly reduced mean levels during the exposure period and increased mean levels with the onset of fibrosis. The initial dilution on exposure can be explained by mucosal hypersecretion but not the sustained increase above the unexposed level when fibrosis is established. This latter is probably associated with the active accumulation of iron which combines with mucopolysaccharides during the formation of asbestos bodies; an analysis of the coating of asbestos bodies is reported by Blount, Holt and Leach (1966).

Four gamma globulins were investigated, gamma a, gamma e, gamma g and gamma m. Two of these, gamma e and gamma m are eliminated as markers on first analysis and it is pertinent to note that whereas gamma e and gamma m are monomeric gamma a and gamma g exhibit polymerisation. Gamma a follows the pattern of transferrin, falling during exposure and rising with the onset of fibrosis. The mean gamma g level does increase on exposure, and although falling with fibrosis, remains above the unexposed level. Whilst this behaviour of gamma g may partly explain the beta/gamma finding (Table 7), it seems more likely that the increase on exposure of this fraction will be found in the beta globulin fraction. For this reason it was decided to try alternative methods of protein fractionation that would achieve better partioning of the beta/gamma globulin fractions. Since the proteins of interest lie in the beta/gamma range, it was decided to investigate fractionation of sputum proteins on a cation exchanger at a pH marginally below 7 in order to retard the release of the globulin components.

2.5.1 Ion Exchange Separation on Cation Exchanger

2.5.1.1 Materials and Method

Sputum prepared as described in Section 2.1.4.

C.M. Sephadex C 50 Pharmacia

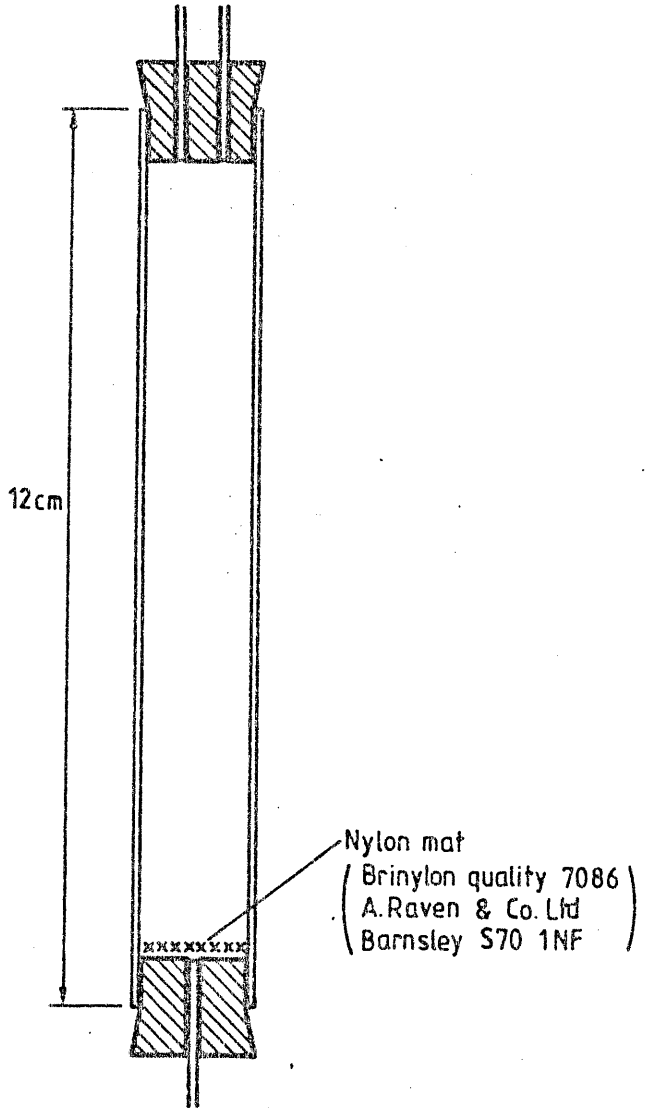
0.05 M sodium acetate - adjusted to pH 6.5 with glacial acetic acid.

U/V monitor Isco model UA 5
Instrument Specialities Co., Lincoln, Nebraska.

10 inch single pen recorder Shandon Southern Ltd., Cheshire.

Fraction Collector Minirac 17000 L.K.B. Ltd.,

Figure 14 The column used for cation exchange separation of sputum

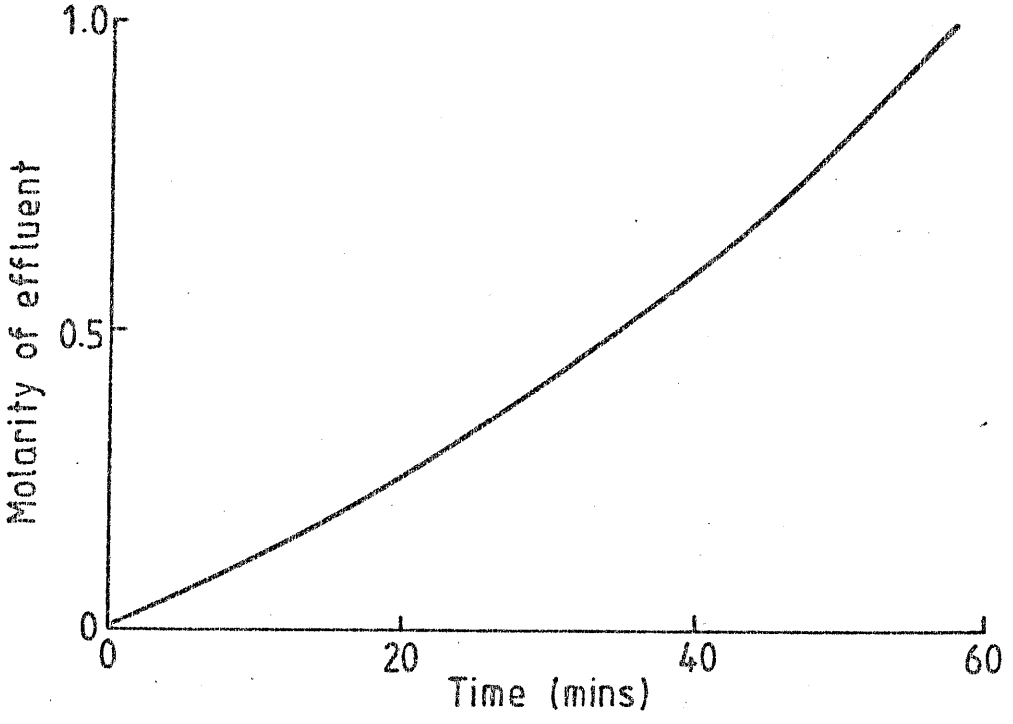


Since, for the majority of sputum samples a total volume of 0.2ml was available for analysis a very small column was required and no suitable commercial product was found. The optimum specifications were found only after several trials. A glass column was made by cutting a 12cm. length from borosilicate glass tubing with an internal diameter 1.5cm. This tube was then steeped in 0.1M hydrochloric acid for seven days to leach out free alkali; it was then irrigated with several litres of deionised water. The column was closed with rubber bungs pierced with stainless steel tubes (No. 19 serum intravenous needles cut square) and a nylon mat cut to fit the base of the column (see Figure 14).

CM Sephadex C50 was equilibrated with the sodium acetate solution by daily changes for seven days. Before use, the supernatant buffer was drawn off the Sephadex and the slurry poured into the vertically mounted column to a bed height of 100mm. Under starting conditions the bed volume was 5ml. and was passed in 4 minutes when the pressure head of buffer was 600mm. An ultraviolet monitor set at 280nm with 0.5 absorbance at full scale deflection was used to read the column effluent and a 10 inch recorder set at 1 volt sensitivity (chart speed 1mm/min), recorded the trace. Fractions were collected in the Minirac fraction collector that was phased in with the recorder.

The apparatus was run under starting conditions for 2 hours to equilibrate. When conditions were steady, 0.1 ml sputum was carefully superimposed on the bed surface and elution carried out until the trace returned to the baseline. A molar sodium chloride gradient was initiated by means of a Sephadex gradient mixer containing 75ml buffer in the first reservoir

Figure 15 The sodium chloride gradient during elution on CM Sephadex C50



and 75ml buffer containing sodium chloride at 1 molar concentration in the second reservoir. At the point of exhaustion of the buffer a further 10ml buffer containing molar sodium chloride was passed through the column to ensure clearance of all protein from the exchanger. As the salt concentration of the eluting buffer increases, progressive shrinkage of the exchanger bed occurs to a final height of 53mm. and the space above the bed surface fills with eluant. In these circumstances the ionic gradient should be non-linear. This was confirmed when the molarity of the eluant was determined by titration against a standard solution of silver nitrate (see Figure 15). When the separation was completed the CM Sephadex C50 was returned to starting conditions by irrigation with starting buffer and then the column was left to stand overnight to equilibrate.

Specimens, representative of each cation exchange fraction, were concentrated by dialysis against polyvinyl pyrrolidine across a Cellophane membrane. Seamless Cellophane tubing ('Visking', Gallenkamp Ltd., London) with a cut-off equivalent to M.Wt. 12,000 for spherical shaped molecules, was cut into 12cm. lengths. After soaking in deionised water, one end of the tube was tightly knotted to form a dialysing sac into which was poured about 10g. polyvinylpyrrolidine powder. The protein solution was placed in a 1 oz. polystyrene container (Sterelin Ltd., Teddington, Middx.) together with a polystyrene bead to prevent total loss of water to the polyvinylpyrrolidine. The dialysing sac was placed in the container (see Figure 17) and dialysis was carried out overnight at +5^{o}C. The resulting concentrates were then stored

Figure 16 Typical trace obtained during the separation of sputum proteins on cation exchanger CM Sephadex C50.

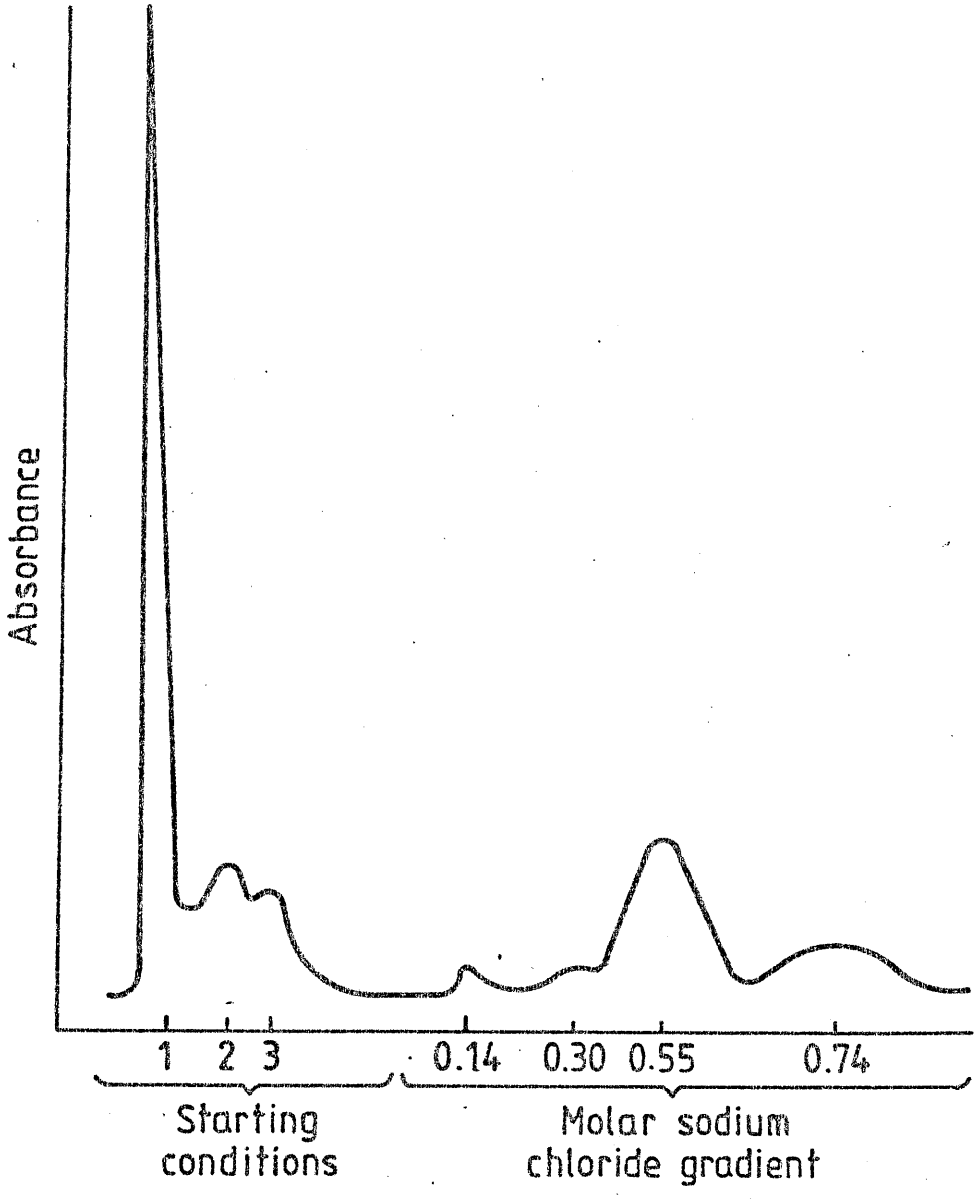
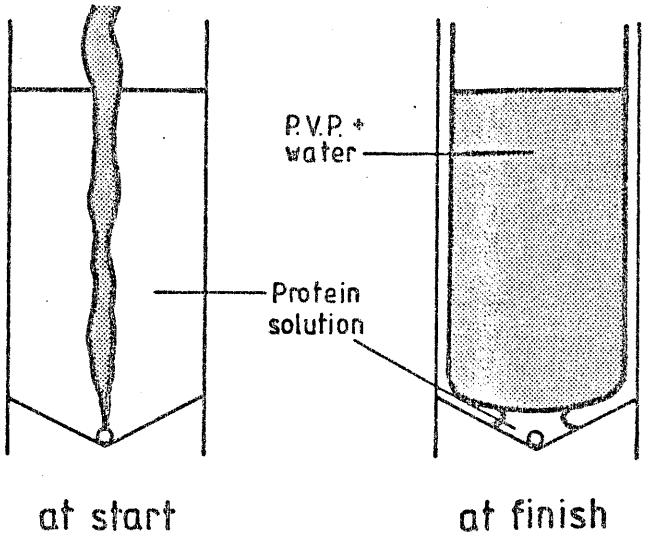


Figure 17 The method used for dialysis of the fractions



at $0^{o}C$ until required for analysis. Each concentrate was examined by electrophoresis and immunodiffusion by the methods described in Section 2.3 and 2.4. Samples from each fraction were also spotted onto two cellulose acetate strips which were then stained by the PAS and the alcian blue/PAS methods.

2.5.1.2 Results

All sputum samples yielded three protein fractions when the loaded column was eluted with the starting buffer, 0.05M sodium acetate. It can be seen from Figure 16 that the recorder trace showed an initial sharp peak of totally unbound proteins followed by two smaller peaks of lightly-bound proteins after which the trace returns to the base-line. The absorbance of both smaller peaks was about 1/10 that of the first peak. Subsequent elution with buffer produced in the gradient mixer, yielded peaks along the gradient at the following molarities:

0.06 0.14 0.23 0.30 0.37 0.49 0.57 0.74

The number of peaks detected on the gradient in any one sputum specimen varied between one and six (see Table 13). Sputa from 6 unexposed, 8 exposed without asbestosis and 5 exposed with asbestosis donors were examined. Analysis by the variance ratio test showed that the distribution of the peaks on the gradient was not significant with respect to donor status at $p = 0.05$, $F_{(Calc)} = 0.3$, $F_{(table)} = 3.5$.

Electrophoresis of the dialysed fractions showed that the complex of unbound proteins forming the first separation peak contained albumin, alpha,beta and gamma globulins.

Table 13 The Separation Pattern of Sputum Proteins on Cation Exchanger CM Sephadex C50

Donor status	Fractions eluted with starting buffer			Fractions eluted on the sodium chloride (0.0 to 1.0 molar)							
	1	2	3	0.06	0.14	0.23	0.30	0.37	0.49	0.57	0.74
Unexposed (6 cases)	6	6	6	3	3	4	1	3	2	3	0
Exposed without asbestosis (8 cases)	8	8	8	4	2	3	4	2	6	2	3
Exposed with asbestosis (5 cases)	5	5	5	1	2	1	1	4	0	1	3

Table 14 Results from Analysis of the Fractions Obtained by Cation Exchange separation of sputum solution (immunodiffusion)

		starting condition			Sodium chloride gradient 0 to 1.0 molar							
Protein	M.Wt.	1	2	3	0.06	0.14	0.23	0.30	0.37	0.49	0.57	0.74
Whole serum	-	+	+	-	+	+	+	+	+	+	-	-
gamma g+a+m	-	+	+	-	-	+	+	-	-	+	-	-
gamma g	160,000	+	+	-	-	-	-	-	-	-	-	-
gamma a	$(160,000)_n$	+	+	-	-	+	-	-	-	+	-	-
gamma m	$(10^6)_n$	+	+	-	-	+	-	-	-	+	-	-
gamma e	190,000	+	-	-	-	-	-	-	-	-	-	-
Prealbumin	55,000	+	-	-	-	-	-	-	-	-	-	-
Albumin	66,300	+	+	-	-	-	-	-	-	-	-	-
Transferrin	76,500	+	+	-	-	-	-	-	-	-	-	-
Haptoglobulin	$(10^5)_n$	+	+	-	-	-	-	-	-	-	-	-
Ceruloplasmin	151,000	+	-	-	-	-	-	-	-	-	-	-
Fibrinogen	341,000	-	+	-	-	-	-	-	-	-	-	-
$beta_1C beta_1A$	?	+	-	-	-	-	-	-	-	-	-	-
$beta_2$ glycoprotein	35,000	+	-	-	-	-	-	-	-	-	+	-
$alpha_1$ acid glycoprotein	40,000	+	+	-	-	-	-	-	-	+	-	-
$alpha_2$HS glycoprotein	49,000	+	-	-	-	-	-	-	-	+	-	-
$alpha_2$ macroglobulin	725,000	+	-	-	-	-	-	-	-	-	+	-

Note: all the fractions contained PAS staining protein

Table 15 Results from Analysis of the Fractions Obtained by Cation Exchange Separation of Sputum Solution (Electrophoresis)

Protein fraction	Location on electrophoresis
Starting condition Peak 1	Albumin, alpha globulin, beta globulin, gamma globulin.
Starting condition Peak 2	$beta_3$/gamma
Starting condition Peak 3	$beta_3$
0.06 Molar	gamma
0.14 Molar	gamma
0.23 Molar	$beta_3$/gamma
0.30 Molar	$beta_3$/gamma
0.37 Molar	gamma
0.49 Molar	gamma, lysozyme
0.57 Molar	gamma, lysozyme
0.74 Molar	gamma

The proteins contained in the second and third peaks obtained during elution with starting buffer occupied the $beta_3$/gamma and $beta_3$ sites. From Table 15, it can be seen that, after electrophoresis, the fractions eluting on the gradient at 0.06, 0.14, 0.37 and 0.74 Molar occupied the gamma location and fractions eluting at 0.23 and 0.30 were present at the $beta_3$ position. The eluates at 0.49 and 0.57 molar yielded the same result; both showed bands at the gamma and the lysozyme positions.

Immunoanalysis of the dialysed fractions showed that all of the prealbumin and gamma e, together with part of each of the other 12 specific proteins sought, were eluted from the column in the unbound protein complex that formed the first peak. The second peak contained all of the fibrinogen, further portions of gamma a, gamma m, $alpha_1$ acid glycoprotein and beta glycoprotein together with all residual albumin, transferrin, haptoglobulin and gamma g globulin that had been left on the column. The third peak obtained with the starting buffer consisted of non-plasma proteins. This is shown in Table 14 where it can be seen that no immunoprecipitate was obtained when the fraction was challenged with antiserum against whole human serum (line 1) and fibrinogen (line 12). All fractions eluting from the column during the sodium chloride gradient contain serum proteins. Unidentified serum proteins were present at 0.06, 0.30 and 0.37 Molar. Gamma a and gamma m were identified in the eluates at 0.14 and 0.49 Molar. Three further proteins were found at 0.49M these were $alpha_1$ acid glycoprotein, $alpha_2$ HS glycoprotein and $alpha_2$ macroglobulin. $Alpha_2$ macroglobulin was also present at 0.57 Molar together with

$beta_2$ glycoprotein.

The cellulose acetate strips stained by the PAS and alcian blue/PAS methods showed that acid and neutral glycoproteins were present in all the eluted fractions.

2.5.1.3 Discussion

The ion exchange method was designed to examine especially those proteins with beta electromobility. Proteins with this characteristic were present in five fractions eluted from sputum on the cation exchange column. Some beta protein was totally unbound to the exchanger and was immediately eluted by the starting buffer in the first fraction. A protein with beta mobility was lightly-bound to the exchanger and this was present in two succeeding fractions that were released by further elution with starting buffer. The last of these two fractions consisted entirely of a nonserous protein with $beta_3$ electromobility. It is probable that the presence of $beta_3$ protein in the preceeding fraction was caused by incomplete partitioning of the lightly-bound proteins during elution from the column. It can be seen in Figure 16 that the three fractions followed too closely upon one another for the trace to return to the base-line between each peak.

Proteins with $beta_3$ mobility were also present in two fractions released from the column during the sodium chloride gradient. These were found in the 0.23M and 0.30M fractions. Elution at two closely-related molarities rather than at a single point, might be due to variations in the density of

surface charge between otherwise similar molecules if the protein exhibits polydispersity (varying degrees of polymerisation) as occurs in all mucus glycoproteins (Reid and Clamp, 1978). The fact that the gradient fractions yielded an immunoprecipitate with anti-whole human serum does not necessarily imply that these proteins differ from the $beta_3$ protein eluted with starting buffer; it may be the same protein covalently linked to a serum gamma globulin as described by Roberts (1974, 1976). Clamp, (1977) considers that such linkages could have important implications for the role and function of antibodies in mucus secretion.

Although the distribution of eluted proteins on the sodium chloride gradient is not statistically significant with respect to donor status, the percentage frequency of detection of the 0.23/0.30M protein shows a trend toward loss of this fraction in sputum from individuals exposed to asbestos without and with fibrosis - unexposed 66%; exposed without asbestosis 50%; exposed with asbestosis 20%. If the complexing of mucus glycoprotein with serum globulins is important in mucus secretion, as suggested by Clamp (1977), the progressive loss of the 0.23/0.30M fraction may explain the reduction in mucus secretion that is a feature of established asbestosis.

The use of cation exchange for the analysis of whole sputum differs from the approaches adopted by other workers. Generally, pooled sputum specimens have been collected and separated into sol and gel phases before analysis by gel filtration. The separation of degraded

sputum fractions on anion exchanger columns however, was reported by Degand, Roussel and Lamblin (1974) and Boat, Cheng, Iyer, Carlson and Polony (1976). A review of published work on bronchial secretions has been made by Creeth (1978). He concludes that plasma proteins are present in sputum through transudation and that albumin, gamma a and gamma g are the most significant plasma proteins in the sputum. Of the non-plasma proteins reported, he considers that secretory gamma a, lactoferrin, lysozyme and amylase are the most significant. None of the studies has reported the isolation of either of the $beta_3$ components found in the present study. Interest in previous research has largely centred on the separation of the neutral fucomucins and the acidic sialo - and sulphomucins and also on the composition of these mucus glycoproteins. Clamp (1977) defines the most important constituent of mucus as a glycoprotein with a molecular weight of 'several million' containing more than half its weight as carbohydrate and in a later paper (Reid and Clamp, 1978), a proposed nomenclature, based on biochemical and histochemical properties of mucus, is suggested. The native glycoprotein is described as having a central polypeptide spine with oligosaccharide units projecting from it in a form similar in shape to a test-tube brush. This constitutes the neutral mucin and, where sialic acid or sulphate groups are terminally attached to the bristles, these are acidic mucins. The third fraction released with starting buffer found in the present study does not fit well into this categorisation. Its discrete fractionation on cation

exchanger and $beta_3$ electromobility indicate a uniformity of structure and its probable association with a serum gamma globulin suggest a physiologically active role in the mucosa.

No specific marker protein for asbestosis has emerged from these investigations but the results do suggest a general pattern of response to the inhalation of asbestos dust. Dust inhalation induces hypersecretion without significant alteration in permeability of the airway mucosa.

Studies of lung ultrastructure and protein transport across the endothelial/epithelial barrier in mouse lung (Schneeberger, 1976) and foetal sheep lung (Strang, 1978), indicate that diffusion of plasma proteins can occur through the interendothelial cell junctions when these are stretched by increased intravascular pressure. The tight junctions between the alveolar epithelial cells, both type 1 and type 2 pneumocytes, form a barrier, the zonulae occludentes, that prevents further movement of protein into the alveolus. Schneeberger (1976), however, suggests that bidirectional transport of protein takes place across the mouse type 1 pneumocyte by pinocytosis and considers that the process is important for delivering small quantities of serum albumin and immunoglobulins to the alveolus. Other researchers postulate that, in the rat lung (Whayne and Severinghause, 1968) and in the dog lung (Staub, Gee and Vreim, 1976) the epithelium of the terminal bronchioles is normally permeable to protein and water and that it is by this route that plasma proteins enter the alveolus. Staub, et al (1976) were able to demonstrate plasma uptake of albumin that was introduced into the fluid-filled lobes of the lungs of anaesthetised dogs.

However, the results from the animal models used to elucidate these transportation routes cannot be extrapolated to the human clinical situation without reservation.

In the human, Clamp (1977) has observed that 'even uncomplicated seromucous secretions contain a large number of other constituents including most serum proteins'. Creeth (1978) attributes the presence of serum proteins in sputum to transudation. It has been shown (Pump, 1974) that damaged alveoli can be found in apparently normal lung at ages between 5 and 66 years. This damage is occasioned by rupture of the continuum of elastic fibrils surrounding the alveoli. The affected alveolus dilates forming a fenestra. The type 1 pneumocytes detach from the damaged wall and are not replaced and in this condition plasma flows into the sac without impediment (Bowden 1981).

It is also possible that the presence of plasma proteins in the sputum from unexposed donors is a consequence of areas of chronically-infected bronchial mucosa. At these sites bacterial cytotoxins cause exfoliation of epithelial cells and altered permeability of the basement membrane.

The significantly lower albumin levels in sputum from exposed individuals without asbestosis indicates that the broncho-alveolar epithelium is largely intact. This is surprising because the presence of chrysotile fibres trapped in continually expanding and contacting alveoli would be expected to damage the type 1 pneumocytes lining and thus initiate type 2 cell proliferation. If this does occur, careful appraisal of the cells present in sputum from exposed individuals without asbestosis might reveal evidence of this in the form of type 2 pneumocytes possibly in association with inflammatory cells.

CHAPTER THREE

The Broncho-Alveolar Secretion as Represented By Sputum

Part Two

The Cytology of the Three Donor Groups

The Broncho-Alveolar Secretion as Represented by Sputum

The Sputum Cytology of the Three Donor Groups

3.1.1 Introduction

Provided that secretion and exfoliated cells from the mouth and pharynx are eliminated with a preliminary mouth-wash and gargle, most of the components of sputum collected by the 'deep cough' method will have arisen from the mucosa lining the trachea and larger bronchi. The sputum specimen usually, however, also contains some elements from the distal airway. This material is probably released by the piston effect induced when the mucus in the terminal bronchioles is pulled out onto the mucociliary escalator during the period of forced exhalation. The Curschman spirals, sometimes found in patients suffering from chronic bronchitis or asthma, are casts formed from progressively inspissated mucus held in the terminal bronchioles but released during violent coughing.

Microscopical examination of the sputum in asbestosis has been confined to the search for asbestos bodies, as confirmatory evidence of exposure, and also for malignant cells when neoplasia is a suspected sequel to exposure. A general appraisal of the cytological picture seen in sputa from patients with a history of exposure to asbestos has not been reported. This is probably because the characteristic, but nonspecific, histological changes seen in asbestosis suggest that such an investigation would yield equally nonspecific findings.

It is generally accepted that, in interstitial lung disorders, an association exists between alveolitis and fibrosis although the causes of this relationship are not understood. Current thinking on this concept is reviewed by Keogh and Crystal (1982) who categorise these lung diseases into two groups according to cell response. The classification is based initially on the predominant cell type present in the alveolar spaces 1) inflammatory cells (neutrophils) and 2) immune effector cells (lymphocytes). Asbestos induced alveolitis is placed in the first group. Two associated papers, (Schoenberger, Gadek and Crystal, 1981 and Shoenberger, Kawanami, Ferrans and Crystal, 1982), provide evidence that, when guinea pigs are subjected to intratracheal injection of chrysotile asbestos, a 'polymorphonuclear alveolitis' occurs within three days and persists for at least six weeks. Proliferation of type 2 pneumocytes in asbestosis as seen in histological sections has been reported by Kuhn and Kuo (1973) and Selikoff and Lee (1978). For these reasons it was considered worthwhile to assess the cytological content of sputum smears from the three donor classes in order to discover whether supportive evidence of damage to the distal airway epithelium can be detected after exposure to asbestos and also to see whether any differences in sputum cytology can be found between the three donor groups.

3.1.2 Materials and Method

Sputum specimens were examined immediately after they were received into the laboratory.. The specimen was transferred to a disposable 4 inch Petri dish and an inspection of the material in the dish was made under a dissecting microscope (Olympus SZ Stereozoom) at x 3.5 to x 160 magnifications. The criteria used for selecting material for smear preparations, in the order in which they were applied, were as follows:

1. bloodstained flecks (indicating probable recent haemorrhage).
2. black specs (indicating probable carbon-containing histiocytes).
3. green streaks in the mucus (indicating altered blood - old haemorrhage).
4. white flecks (indicating probable tissue fragments)

In the absence of any of these criteria a sweep was taken through the mucus in the Petri dish.

The chosen material was extracted from the specimen with a disposable wood applicator ('cherry stick' type) which was then drawn across a microscope slide. Each smear was fixed immediately after it was made by placing the slide in a solution comprising equal volumes of diethyl ether and 74 O.P. industrial spirit and the slides left for 20 minutes before staining. Nine slides were prepared from each specimen. The sputum remaining in the Petri dish was then returned to its original container and stored at $+5^{\circ}C$ until processed for protein analysis.

Six smears were stained from each specimen using the following methods:

1. Papanicoloau stain, (Taylor, 1967).
2. Jenner Geimsa stain, (Taylor, 1967).
3. Gram stain (Taylor, 1967),
4. Perl's Prussian blue stain (Drury and Wallington, 1968),
5. Periodic acid/Schiff (PAS) stain (Pearse, 1972),
6. Unna Pappenheim stain (Jordan and Baker, 1955).

All smears were first scanned at x 100 magnification to assess the general cell content and to locate areas of interest in the smear which were then marked with glass-writing ink. These areas were later scrutinised at x600 magnification and, where necessary, further examined at x 1000 using an oil immersion lens. A protocol was prepared and the cells in a smear catalogued initially according to accepted morphological criteria and recording the presence or absence of each cell type during the examination of the Papanicoloau-stained smear. Supplementary information concerning the cells was obtained from examination of the remaining stained smears. The Jenner Geimsa stained smears yielded confirmation of the types of blood cells seen in the Papanicoloau-stained smears. The Gram stained films were used to identify microorganisms present in the sputa. The presence of ferric iron in histiocytes was detected by means of the smears stained by Perl's Prussian blue method. PAS stain was used to identify mucin in columnar goblet cells and Unna Pappenheim's stain was used to examine the nucleoli in the cell nuclei.

3.1.3 Results

Epithelial Cells Seen in the Smears

Superficial squamous cells were present in all the smears examined.

Ciliate columnar cells were detected in 15% of smears from unexposed donors and 32% of smears from donors in both exposed groups.

Goblet columnar cells were not seen.

Metaplastic (columnar) cells were not seen in smears from unexposed donors but were present in 14% and 5% of smears from the exposed with asbestosis and exposed without asbestosis groups respectively.

Distal airway epithelial cells (Type 1 and Type 2 pneumocytes) were absent in the smears from the unexposed group. Type 1 pneumocytes were identified in 50% of smears from both exposed groups but the detection frequency of Type 2 pneumocytes differed between the two exposed groups - 27% in the exposed with asbestosis and 18% in the exposed without asbestosis groups.

Blood Cells and Lymphoreticular Cells

Erythrocytes were not found in smears from the unexposed group but were present in 55% of smears from the asbestosis group and 14% of smears from the exposed without asbestosis group.

Neutrophils were seen in 77% of smears from the unexposed group; in 86% of smears from the exposed with asbestosis group and 77% of smears from the exposed without asbestosis group.

Lymphocytes were present in 23% of sputum smears from the unexposed donors and 64% and 73% of sputum smears from asbestotic and exposed without asbestosis donors respectively. Plasma cells and eosinophils were absent in smears from unexposed donor sputum but plasma cells were present in 10% and eosinophils were present in 14% of sputum smears from both the exposed donor groups.

Histiocytes were present in 84% of sputum smears from unexposed donors and 86% and 91% of sputum smears from exposed donors with asbestosis and without asbestosis respectively. Histiocytes containing carbon were found twice as frequently in sputum smears from exposed donors compared with unexposed donors. Ferric iron was not found in histiocytes present in sputum smears from unexposed donors but ferric iron was present in histiocytes in 10% of sputum smears from exposed donors. Histiocytes containing intracellular asbestos bodies and others containing chrysotile fibre were found only in sputum smears from exposed donors with asbestosis.

Extracellular chrysotile fibre was found in 64% of sputum smears from asbestotic donors and in 55% of sputum smears from donors exposed without asbestosis. Extracellular asbestos bodies were found in 27% of sputum smears from asbestotic donors and 5% of sputum smears from donors exposed without asbestosis. Asbestos bodies stained positive for ferric iron while the free chrysotile fibre did not.

Numerous microorganisms of several types were present in all smears examined and also debris from damaged somatic cells.

A summary of these results is given in Table 16.

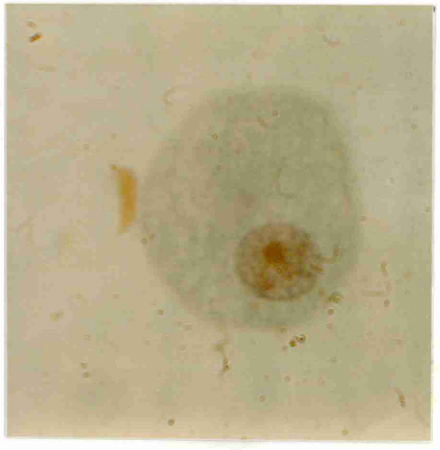
Table 16 Results of Analysis of Cells in the Sputum Smears

	Unexposed to asbestos	Exposed without asbestosis	Exposed with asbestosis
Superficial squamous cell	100	100	100
Ciliate columnar cell	15	32	32
Goblet columnar cell	8	0	0
Metaplastic cell	0	5	14
Type 1 pneumocyte	0	50	50
Type 2 pneumocyte	0	18	27
Erythrocyte	0	14	55
Neutrophil	77	86	77
Lumphocyte	23	73	64
Plasma cell	0	10	10
Eosinophil	0	14	14
Histiocyte	84	91	86
with carbon	38	91	82
with iron	0	9	10
with chrysotile	0	0	14
with asbestos body	0	0	18
Chrysotile fibre		55	64
Asbestos body	0	5	27
Curschman spiral	0	3	0
Number of cases	13	22	22

Cell Frequency %

Figure 18 The cells of the distal airway epithelium

Type 1 (membranous) pneumocyte left of picture



Type 2 (granular) pneumocyte

Type 2 (granular) pneumocyte showing multinucleation

Lung histiocytes (for comparison)

3.1.4 Discussion

Sputum specimens from all three donor classes contained ciliated columnar cells from the bronchial mucosa but these cells are found with twice the frequency in sputa from donors exposed to asbestos compared with sputum from donors in the unexposed group. In addition, the presence of metaplastic cells in sputa from exposed donors, 5% and 14% without asbestosis and with asbestosis respectively, suggests that these cells have arisen from patches of squamous metaplasia which, in turn is indicative of a chronic inflammatory reaction.

Although Kikkawa and Yoneda (1973) have reported type 2 pneumocytes in Papanicoloau-stained smears, identification of these cells remains controversial. There is little doubt that the cells regarded as large, foamy macrophages (large lung histiocytes) by some workers, are the same cell type considered in this study to be type 2 pneumocytes. When these cells are present in sputum smears, they can be differentiated from normal lung histiocytes by their larger size, less dense cytoplasm, large eccentrically-placed nucleus containing a prominent peripherally positioned nucleolus and absence of ingested particles. See Figure 18.

Retained chrysotile fibre causes exfoliation of type 1 pneumocytes, (see Figure 18). This is a cell which is also reported with some reservation since it appears to be unrecognised by other workers although it can frequently be seen in bronchial washings obtained from patients with clinically-established pneumonitis.

The high frequency of neutrophils, 77%-88%. in all donor classes contrasts with the results for lymphocytes which are twice as frequently found in the exposed classes compared with unexposed donors. This increase may be associated with the presence of plasma cells and eosinophils; neither of these cells were found in sputum from unexposed donors. The presence of ferric iron in histiocytes also appears to be associated with exposure to asbestos. Histiocytes containing intracellular chrysotile and asbestos bodies appears to be associated with fibrosis since none was found in sputa from the exposed donors without asbestosis.

Extracellular fibre was present in sputa from both exposed classes but asbestos bodies were five times more frequently found in sputum from asbestotic donors. The finding of asbestos bodies in sputa from asbestotic donors has been well documented but Selikoff and Lee (1978) consider that, although it is a striking and potentially protective phenomenon, 'asbestos bodies are something of a side issue insofar as the disabling pathological process is concerned.' Modin et al (1982) consider that asbestos bodies indicate a significant load of asbestos in the lungs.

The general picture emerging from this cytological analysis indicates that exposure to chrysotile dust causes damage to both the middle and the distal airway mucosa and also induces a response by lymphocytes, plasma cells and eosinophils. When fibrosis is established the histiocytes contain ferric iron and asbestos bodies are more frequently found.

3.1.5 Note on Sputum Production

An ability to produce sputum on demand by the deep cough method and in the apparent absence of inducing agents such as dust in the atmosphere or other stimulators of bronchial secretion, is viewed by clinicians as indicative of probable airway pathology. Brewis (1975) gives a primary parameter of chronic bronchitis as cough with the production of sputum on most days during three months of the year for at least two years.

It is probable that in health only a few intact cells are exfoliated from the distal airway epithelium and the number of neutrophils and histiocytes in the secretion at the air/mucus interface is also small when no external stimulus is present. It follows, therefore, that the sputum donors in the current study who were unexposed to asbestos cannot be regarded as a normal group in respect of cells found in sputum. In order to compare the cytological findings in the study with the cytology arising from the airway mucosa of apparently normal individuals a small series of normal males, who had been subjected to bronchial lavage, were investigated.

3.2 Bronchial Lavage

Seven apparently normal males were subjected to bronchial lavage as described in Section 1.2.3.5.

3.2.1 Method of Processing the Saline Suspension

The volume of wash recovered was measured and 0.25ml of 0.1% pronase in Hank's buffered saline solution were added/ml. saline suspension. The container was placed on a

mixer (Denley Spiramix 5, Denley-Tech. Ltd., Sussex) for one hour at room temperature. Nucleated cell counts were made according to Whitby and Britton (1953) for white blood cells except that 0.5ml suspension was diluted with 0.5ml. of the white cell diluting fluid. The remaining sample volume was centrifuged at 200g for ten minutes and smears were prepared from the deposit. All smears were fixed and stained as described for sputum, Section 3.1.2.

3.2.2 Results

The age range of the individuals comprising the series was between 26 and 78 years and the volume of saline wash recovered ranged between 0.1ml and 31ml. The total number of nucleated cells found in the specimens was between 0.1×10^6 and 3.9×10^6.

Superficial squamous cells were present in 17% and ciliated columnar cells were present in 86% of the specimens. No distal airway epithelial cells were found. All specimens contained blood cells (erythrocytes, neutrophils and lymphocytes) and also histiocytes.

3.2.3 Discussion

Keogh and Crystal (1982) using this technique, estimate that the method samples about 10^6 alveoli and that 50 to 70% of the 100 ml saline used is recovered. The cell content of the recovered wash, 10^7 cells, is given as 90% macrophages and 10% lymphocytes in normal non-smokers while the finding in normal smokers is given as 78% macrophages, 7% lymphocytes and

Figure 19 The Results from Bronchial Lavage (Seven Normal Males)

	Range	Mean	s.d.
Age (years)	26-78	53.14	19.03
Volume recovered (ml)	7-31	17	7.96
Total nucleate cells ($x10^6$)	0.1-3.9	1.46	1.24

	Cell Frequency %
Squamous	71
Ciliate columnar	86
Histiocyte	100
Neutrophil	100
Lymphocyte	100
Erythrocyte	100

5% neutrophils. No reference is made to epithelial cells. These figures indicate a remarkable degree of precision for a technique that contains two major technical difficulties. The method has been in use in this Cytology Unit since January 1981 and about 200 patients have subjected to the procedure. A technical difficulty quickly encountered was that mucus present in the saline washings coagulated thus preventing the uniform distribution of cells in suspension something not referred to in reported work. Attempts to achieve mucolysis without damage to cells were unsuccessful using dithiothreitol and collagenase dispase. After further trials it was found that pronase was a satisfactory mucolytic. The pronase was prepared as a 0.1% solution in Hank's buffered saline. 0.2ml for each ml of cell suspension was added and the mixture was incubated for 30 minutes at 37^{o}C. It was also found that the distribution of cells on the slides was random and differentials were not reproducible even when 1000 cells were classified. Other results from normal subjects in this study are significantly different from those quoted by Keogh and Crystal (1982). Carbon-containing histiocytes were present in all specimens together with neutrophils and lymphocytes in about equal numbers. Erythrocytes were also always found.

The results obtained in the current study show that the technique, as practised in the Cytology Unit at St. Luke's Hospital, traumatises the normal mucosa. The squamous cells are probably carried down the airway on the outer casing of the bronchoscope whilst the erythrocytes almost certainly originate at the point where the canula is extruded from the

bronchoscope prior to irrigation. An interesting finding is that, in those washings containing plaques of ciliated columnar cells, the cilia were motile and exhibited synchronous rhythm. This coherent motility was retained for at least 24 hours after collection when the specimen was stored at +5^{o}C. Unfortunately only two patients suffering from asbestosis have been examined during this study.

CHAPTER FOUR

Water Uptake by Chrysotile During Inhalation

4.1 Water Uptake by Chrysotile During Inhalation

4.1.1 Introduction

In view of its hydrophilic property chrysotile fibre might take up water during inhalation and before making contact with the airway mucosa. The following investigation was designed to assess the amount of water likely to be taken up during this period.

Inhalation and the succeeding post-inhalation pause occupy 1.94 seconds of the respiratory cycle in the normal, resting adult. During this period, under industrial working conditions, particulates in dusty aerosols are carried along the bronchial tree in an atmosphere of increasing humidity and on a temperature gradient ranging from about 20°C to 37°C. The airway currents are complex; the flow-rate of inhaled air decays from about 200 cm/sec. to zero and turbulence, generated at each branching of the bronchial tree, reduces the airstream in the distal airway to low-velocity eddies (Teirstein, 1978). Turbulence is probably a major factor in the removal of suspended particles as the air streams against the mucus coating the ciliated epithelium of the bronchus. Chrysotile fibre, subsequently retained, could take up water during its passage before making contact with the mucosa.

The anatomical complexity of the bronchial tree and the lightness of sieved chrysotile particles preclude the construction of an experimental replica. The mathematical model devised by Weibel (1963) as used for most calculations, is based on the concept of regular, dichotomous branching and

progressive narrowing of the airway. He included further proposals in his paper to take account in part of anatomical variations but neither this alternative nor the one later proposed by Horsfield (1979) is a satisfactory basis for a practical model.

As a first approximation, therefore, it was decided to measure the amount of water taken up by sieved chrysotile when it is exposed to air saturated with water vapour at 37°C. The investigation should yield results higher than would be obtained under in vivo conditions because, for part of the inhalation period of 1.74 seconds the air in the respiratory pathway is at a temperature less than 37°C and it is not saturated with water vapour. A further variable is that, in working conditions, the respiratory cycle may be shortened thus reducing the period of exposure to air saturated withwater vapour at 37°C.

The accuracy of the method is obviously limited. Each weighing necessitates the removal of the chrysotile from the moist chamber for periods of 30 to 45 seconds. This greatly exceeds the physiological time scale (1.74 seconds) but the magnitude of this error can be assessed by continuously monitoring the weight lost by sieved chrysotile that has been previously equilibrated in air saturated with water vapour at 37°C on transfer to dry room conditions.

Figure 19 Method used to expose chrysotile to saturated water vapour

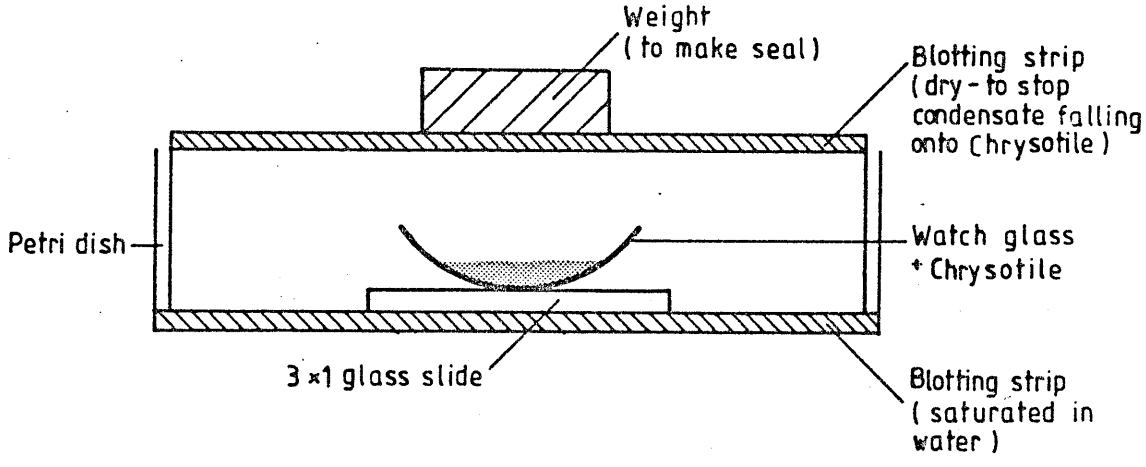
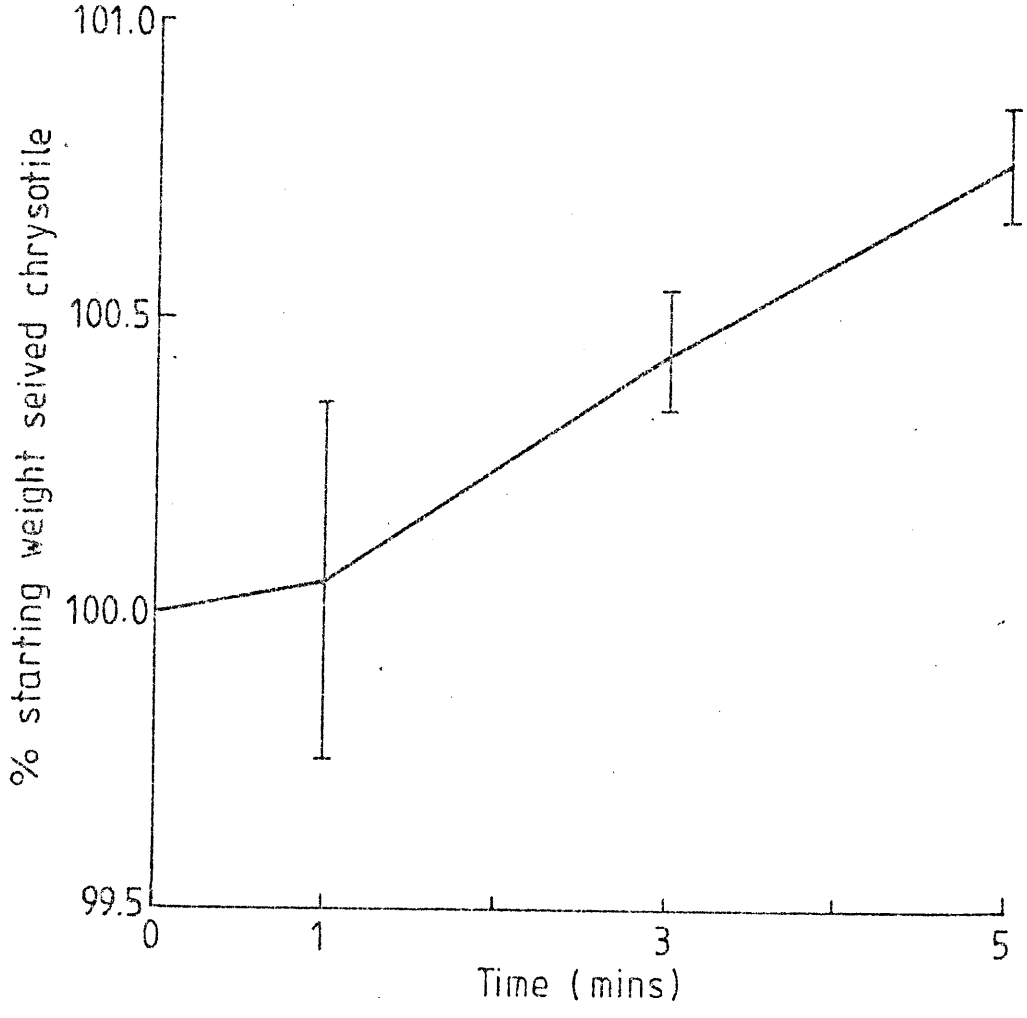


Figure 20 Water uptake by sieved chrysotile from air saturated with water vapour at 37°C.



Time (mins)	0	1	3	5
Mean uptake (%)	0	0.05	0.45	0.78
Standard deviation	-	0.31	0.10	0.10

Correlation coefficient (time : mean uptake) = 0.99

$r_{(table)}$ = 0.99

(p = 0.01)

4.1.2 Materials and Method

Sieved chrysotile prepared as described in Section 1.2.5.

Moist chamber - a 4 inch Petri dish was used as shown in Figure 19.

Balance - Stanton aperodic Model AD2 accuracy ± 0.1 mg.

A watchglass containing about 200mg. sieved chrysotile was left to equilibrate to constant weight in a dry room at constant temperature. It was then transferred to an airtight moist chamber at 37°C and reweighed at timed intervals. Maximum water uptake under these conditions was determined by allowing the chrysotile to remain in the moist chamber until constant weight was obtained. The watchglass containing the chrysotile was then returned to the balance at room temperature. By continuously monitoring the weight of the watchglass and chrysotile the rate of loss of water was measured. The percentage gain or loss in weight of the chrysotile at the timed intervals was then calculated.

4.1.3 Results

In the test conditions sieved chrysotile required more than 20 hours to reach maximal water uptake, +7.6 (±3.3)% of starting weight. The weight gain after 60 seconds exposure to air saturated with water vapour at 37°C was +0.05 (±0.31)%, see Figure 20. From the graph it can be seen that the % weight gain after the first 1.7 seconds exposure (the inhalation period) was +0.002 (±0.01)%.

When the sieved chrysotile in the steady state was transferred from wet air at 37°C to room temperature, the rate of loss of water conformed with the expression:

Figure 21 The rate of water loss from sieved chrysotile on transfer from wet air at 37°C to room temperature (20-0.5°C)

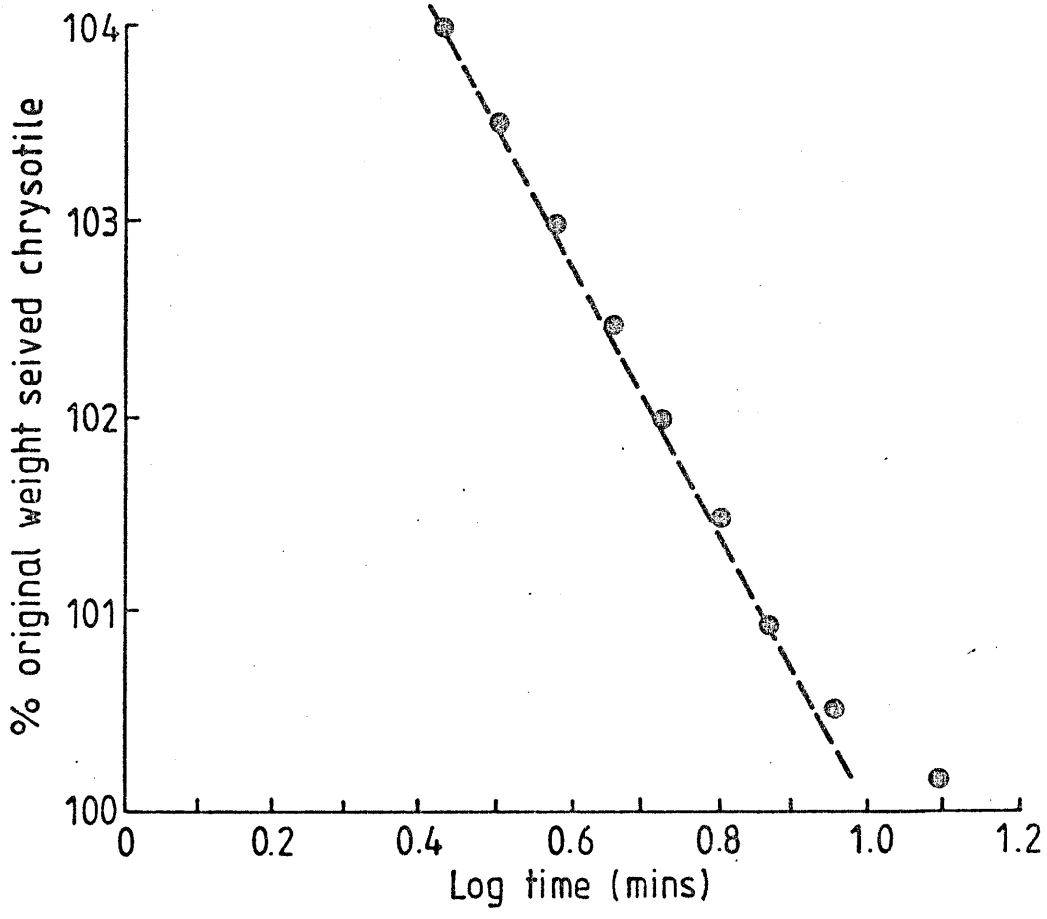
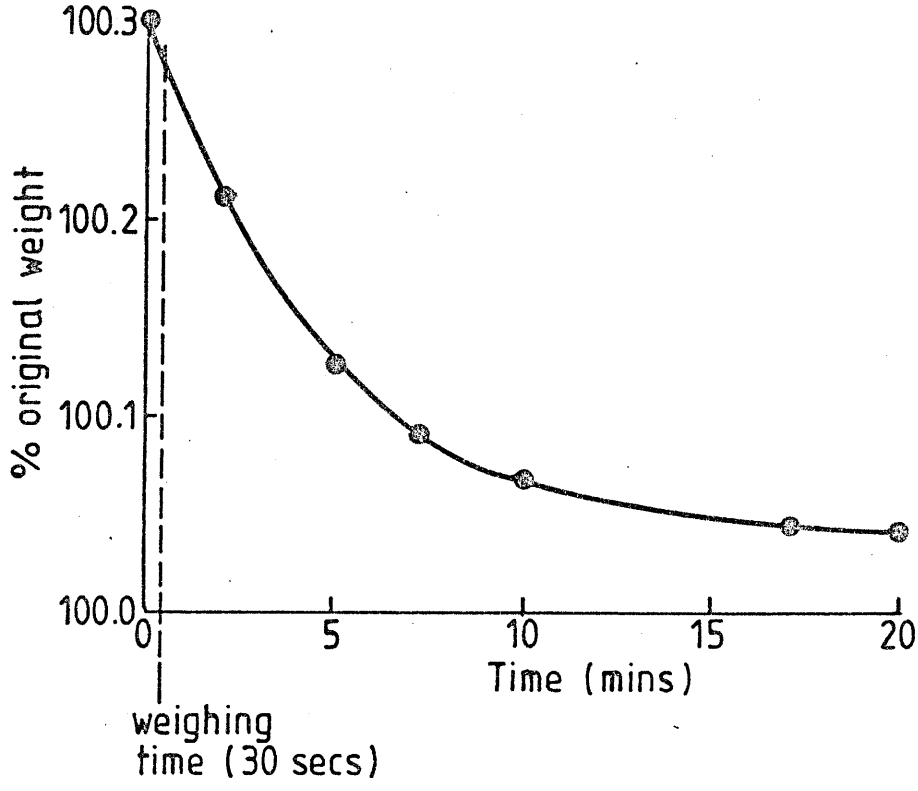


Figure 22 The rate of loss of water by sieved chrysotile below 100.3% starting weight



Rate of loss of water = f $\log_{10}$ time (minutes),
See Figure 21, until the weight had fallen to 100.3% of the starting weight. At 100.3% of starting weight the rate of loss decreased progressively, see Figure 22 , requiring 20 minutes to reach 100.05% of the starting weight.

Table 18 shows the results obtained when the room temperature was 21°C; similar experiments at room temperatures of 19°C and 22.5°C produced only minimal differences in the results.

4.1.4 Discussion

In spite of the relatively crude methods that had to be used for this investigation, some important results were obtained. The critical period of water uptake from water vapour by chrysotile is during the first two seconds; approximately equal to the inhalation time. This investigation shows that, when sieved chrysotile is exposed to air saturated with water vapour at 37°C, it experiences a maximum weight gain of 0.01% and a mean gain of 0.002%. The correction curves were constructed to assess errors incurred by removing the chrysotile from saturated water vapour to air at room temperature for weighing purposes. It can be seen from the Figures 21 and 22 that no measurable loss of water can have occurred during the critical period.

The results show that the magnitude of water uptake by chrysotile during inhalation is unlikely to affect the surface characteristic of the chrysotile fibre before it makes contact with the airway mucosa. It was therefore

Table 18 Water Uptake by Sieved Chrysotile

	Chrysotile weight (mg)	% weight gain
At start	269.6	0
After 1 hour	269.6	0
Transferred to moist chamber at 37°C		0.2
After 1 min.	270.2	0.2
3	270.9	0.4
5	271.8	0.8
10	273.3	1.4
15	274.1	1.6
30	274.4	1.7
45	274.6	1.8
95	276.3	2.7
164	279.2	3.6
274	281.4	4.4
319 ca 5.3h.	283.7	5.3
48 h.	294.5	9.2
49 h.	294.5	9.2
Transferred to balance at 21°C		
After 4 min.	288.8	7.1
5	286.4	6.2
6	285.0	5.7
7	283.8	5.3
8	282.0	4.6
9	281.0	4.2
10	280.1	3.9
11	278.2	3.2
12	277.6	2.9
14	277.3	2.8
15	275.4	2.2
16	274.0	1.6
17	273.4	1.4
18	272.8	1.2
19	272.2	1.0
20	271.6	0.7
21	271.4	0.6
24	270.8	0.4
25	270.5	0.3
28	270.3	0.3
30	270.2	0.2
35	270.0	0.1
37	270.0	0.1
41	270.0	0.1
49	269.9	0.1
50	269.9	0.1
56	269.8	0.1
63	269.8	0.1
81	269.8	0.1

decided to continue the study by placing sieved chrysotile directly into bronchial secretion for the investigations.

CHAPTER FIVE

The Interaction Between Bronchoalveolar Secretion and Chrysotile

The Interaction Between Bronchoalveolar Secretion and Chrysotile

5.1 Introduction

The aim of the following investigations was to discover how quickly inhaled chrysotile fibre takes up water and protein after deposition on the airway mucosa and whether the interaction between fibre and secretion reaches a steady state.

When chrysotile settles on the mucosa the immediate consequence may be that:

1. The fibre takes up water and protein rapidly from the secretion at its immediate location and, by so doing, is drawn into close proximity with the underlying epithelium.

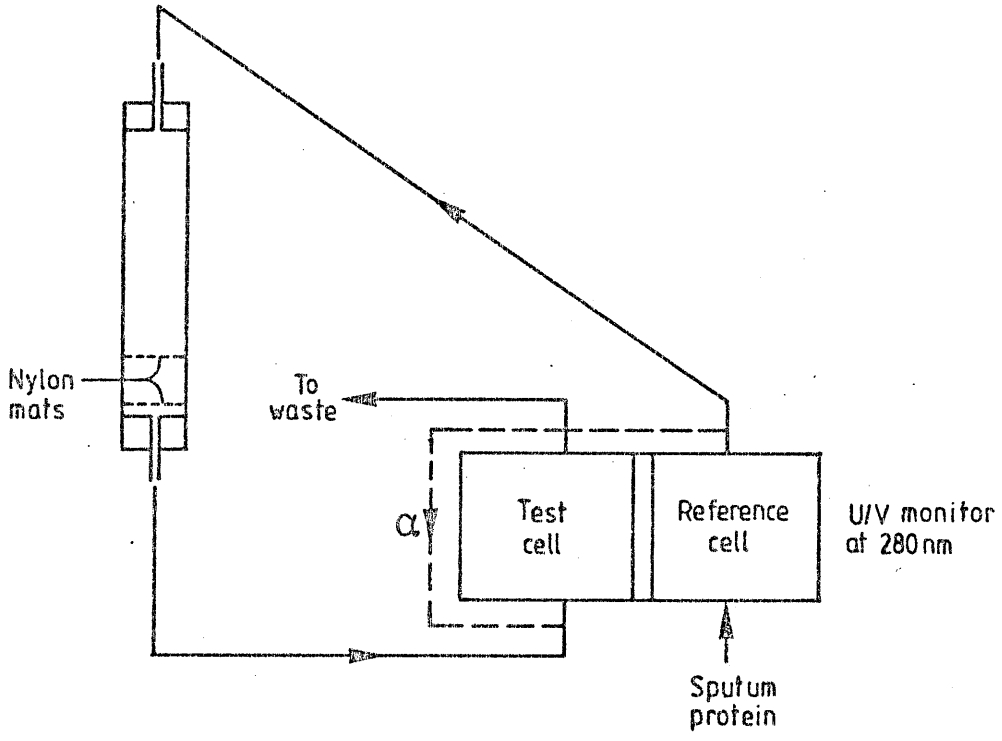
or,

2. The initial coating of secretion on the fibre reduces frictional forces thus lubricating the fibre and enabling it to move freely in the airway secretion.

If the *in vitro* sequence of events can be established, it may indicate which of the two possibilities is more likely to occur *in vivo*. In the first case the expected response would be an immediate uptake of water followed by a more gradual process of protein adsorption whereas the second possibility would be more likely to require concomitant uptake of water and protein.

No published work was found on this topic. Two experimental models were examined; one involved a continous flow of sputum over sieved chrysotile while in the second model a fixed volume of sputum was recycled over the chrysotile until conditions were stable.

Figure 24 Circuit diagram of continuous flow experiment



5.2 Continuous Flow Experiment

5.2.1 Materials and Method

Sieved chrysotile, in dry room condition, was irrigated with a continuous flow of sputum solution at 37°C from an unexposed donor and the optical densities of the afferent and efferent flows were monitored at 280nm in an absorbance monitor (ISCO Model UA-5, Instruments Specialities, Lincoln, Nebraska). The change in absorbance was recorded on a phased-in recorder (Auto-graph S Recorder, Shandon Southern Ltd., Camberley, Surrey).

The apparatus was assembled as shown in Figure 24. A known weight of sieved chrysotile was placed between the nylon mats and the column was incubated at 37°C. Sputum was prepared as described in Section 2.1.4 and diluted to 200 mg, % total protein with 0.1 M sodium acetate. The sputum solution was warmed to 37°C and some was used to prime the apparatus along the "short-circuit" route (labelled 'a' in figure 24). When the recorder trace was steady, the flow of sputum solution was switched through the column and allowed to flow until the solution was exhausted.

5.2.2 Results

When the sputum solution was switched from the short circuit path and allowed to flow through the chrysotile, the trace showed that an increase in absorbance of the efferent sputum solution occurred within one minute. The trace continued as a twin-crested peak followed by a slow fall in

Figure 25 To show trace from continuous flow experiment

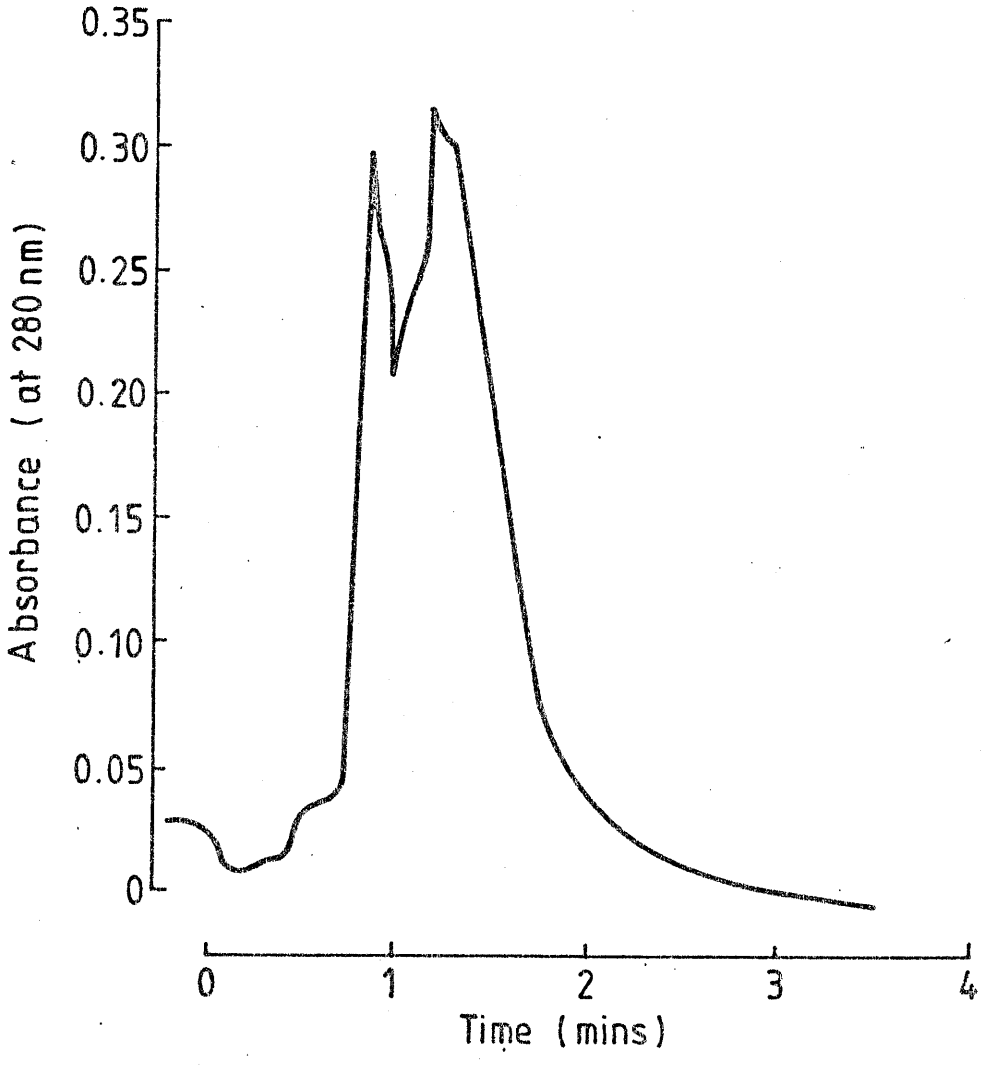


Table 18 Results from the Continuous Flow Experiment

Weight of chrysotile (mg)	Absorbance Increase at 280 nm.	Hydration time (min)
2.5	0.07	0.5
9.5	0.15	2.0
13.5	0.25	3.0
16.5	0.29	4.0

absorbance until the supply of sputum solution was exhausted, see Figure 25. The amplitude and area of the peak varied when different weights of chrysotile were used (Table 18). From these results, it was found that both the maximum increase in absorbance and the time required for the trace to return to its starting absorbance level correlated with the weight of sieved chrysotile used:

For weight of chrysotile and increased absorbance

$$r_{(calc)} = 0.978 \qquad r_{(table)} = 0.959 \qquad p = 0.01$$

For weight of chrysotile and time

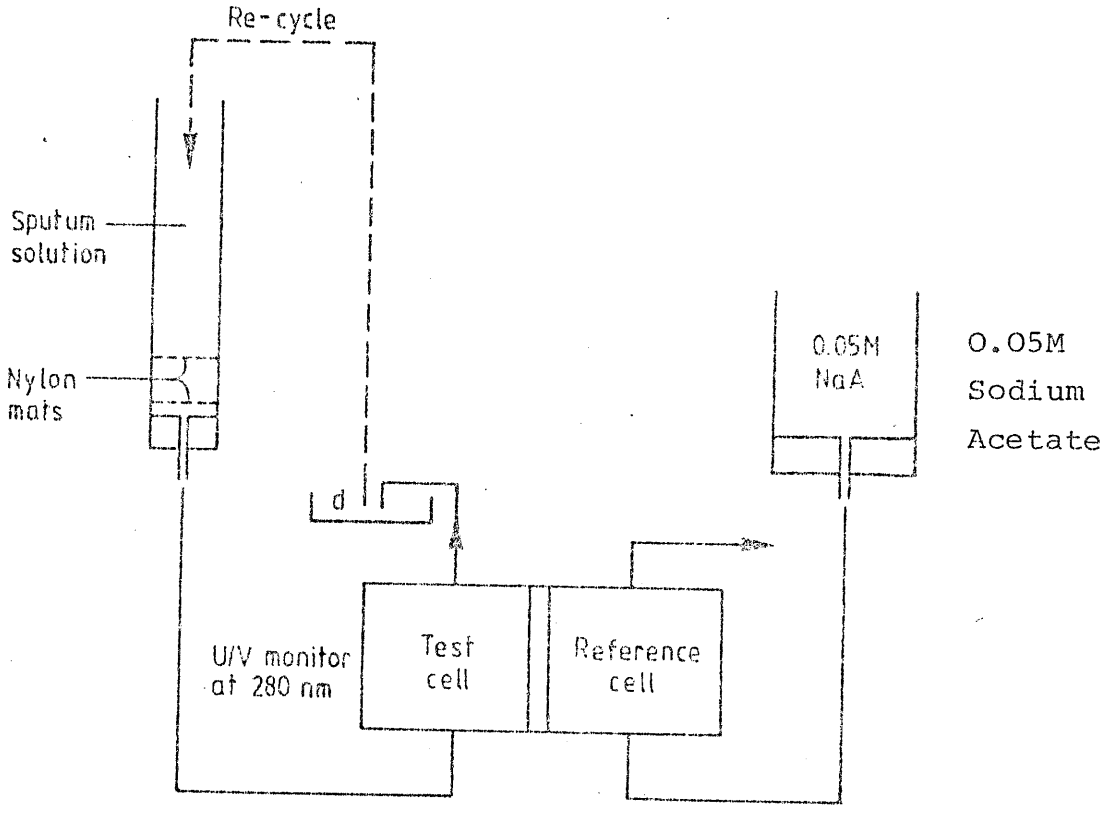
$$r_{(calc)} = 0.995 \qquad r_{(table)} = 0.991 \qquad p = 0.001$$

5.2.3 Discussion

When inhaled chrysotile is deposited in the airway mucosa, it can reasonably be assumed that the fibres are dissociated from one another and that an excess of secretion is available to surround the particle. The use of comparable weights of chrysotile in an *in vitro* model was precluded by the practical difficulties attendant upon measuring and handling such small weights of chrysotile. In the present investigation the volume of sputum required for the weights of chrysotile used was a limiting factor. Sputum from an unexposed donor was diluted to 200 mg. % total protein to approximate to the lowest values found for sputum total protein, See Table 2, Section 2.2.2. This increased the volume of sputum solution available for study.

The initial increase in absorbance of the efferent sputum was probably caused by the removal of water from the sputum by the chrysotile. This is substantiated by the correlation

Figure 26 Circuit diagram for recycling experiment



between the weight of chrysotile and the magnitude and duration above the starting level absorbance. The fact that absorbance continued to fall suggests that protein was being taken up by the chrysotile progressively until all the sputum solution had been used.

Because of the relatively large volume of sputum required for each analysis, further investigation of this technique was not pursued. The results, however, do suggest that the immediate consequence of contact between sieved chrysotile and sputum is an uptake of water followed by an uptake of protein by the chrysotile.

5.3 Recycling Experiment

5.3.1 Materials and Method

The apparatus used in the continuous flow experiment was modifed as shown in Figure 26. The test cell circuit was primed with 5 ml of sputum from an unexposed donor the sputum having been prepared as described in Section 2.1.4. The flow rates of the buffer solution in the reference cell and the sputum in the test cell were synchronised. As the sputum in the reservoir became depleted, it was replenished from the effluent in the collecting vessel (labelled 'd' in Figure 26). After steady conditions had been observed for 15 minutes, a known weight of chrysotile between two nylon mats was added to the test column. The nylon mats were used to prevent the chrysotile fibre from floating free in the circuit. A recorder trace was obtained showing changes in absorbance as in the continuous flow experiment.

Table 19 Results from the Recycling Experiment

Run Number	1	2	3	4		
Weight of chrysotile (mg)	0.33	0.80	1.00	1.10		
Time (min.)					Mean	±s.d.
0	1.18	1.60	1.45	100	-	
0.5	1.24	1.64	0.36	1.49	103.7	1.2
1.0	1.19	1.55	0.27	1.46	102	1.7
2.0	1.18	1.55	0.25	1.42	98.3	1.5
3.0	1.18	1.54	0.24	1.42	98	0
4.0	1.18	1.54	0.23	1.42	98	2.0
5.0	1.16	1.54	0.23	1.42	97.3	1.2
7.5	1.16	1.57	0.23	1.42	98	0
10.0	1.15	1.57	0.21	1.40	97.3	0.6
12.0	1.14	1.57	0.21	1.40	97.3	0.6
15.0	1.15	1.58	0.20	1.40	97.3	0.6
20.0	1.17	1.58	0.18	1.43	99	0
25.0	1.18	1.60	0.16	1.44	99.7	0.6
30.0	1.18	1.60	0.16	1.45	100	-
35.0	1.18	1.60	0.16	1.45	100	-
	absorbance at 280 nm				% starting absorbance	

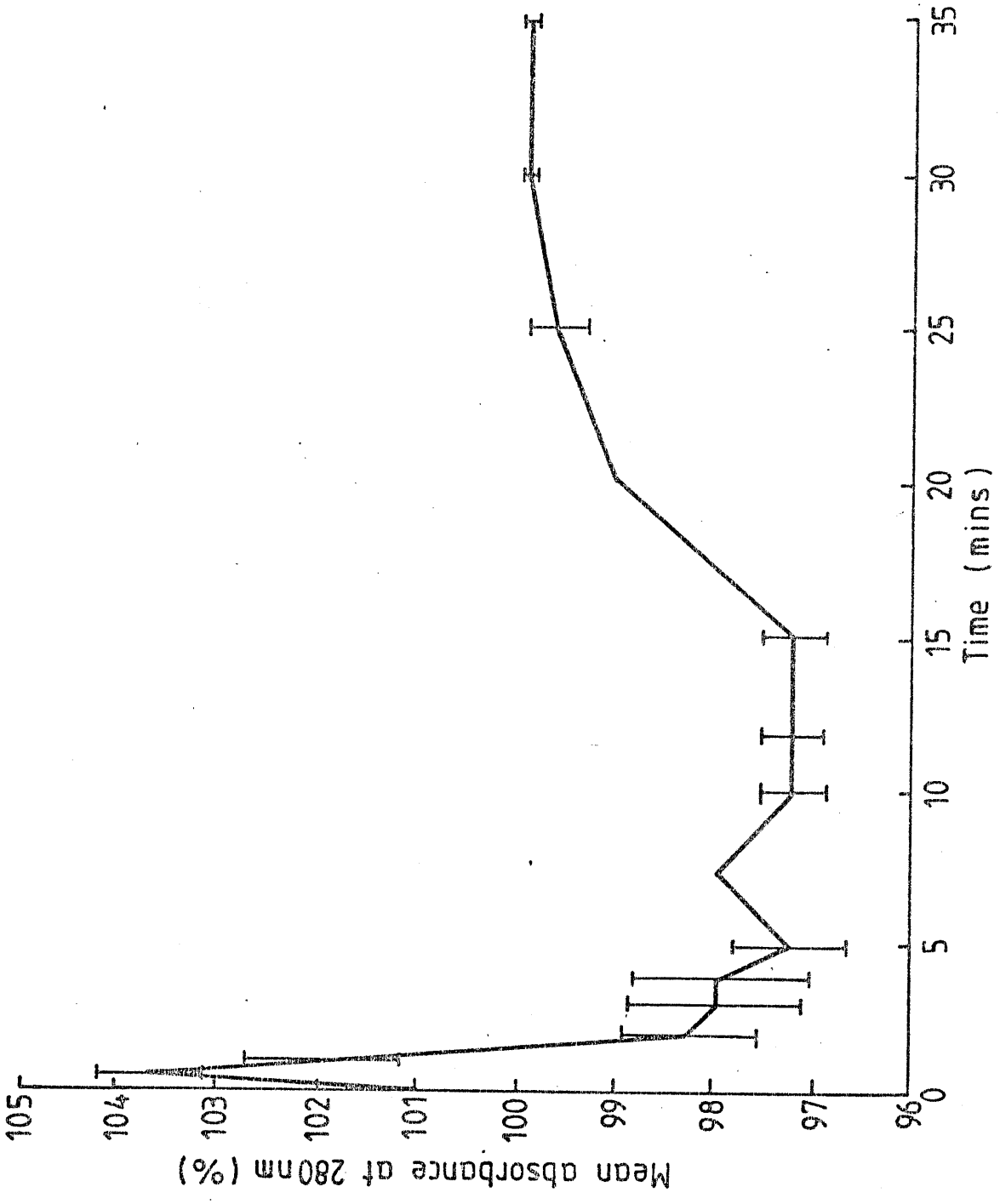


Figure 27 To show change in mean absorbance ± s.d. with time recycling experiment

5.3.2 Results

Four runs were made in the apparatus and during each run a fall in the flow rate through the test cell occurred. This was associated with swelling of the sieved chrysotile that appeared as a gelatinous matrix after 35 minutes immersion in sputum solution.

In runs 1 and 2 the recorder trace returned to the starting absorbance level after 25 minutes and remained steady for the following ten minutes. In run 4 the trace returned to the starting absorbance level after 30 minutes and remained steady for the next five minutes. In run 3 the trace had not returned to starting absorbance after 35 minutes. The results are given in Table 19 and also the mean $\pm$ s.d. % starting absorbance for runs 1, 2 and 4. A graph of these results is shown in Figure 27.

5.3.3 Discussion

On first consideration, it was not thought that the trace would return to the starting level for absorbance when the steady state was reached since both water and protein are taken up from the sputum solution. The probable explanation is that the amounts of water and protein taken up are relatively small in runs 1, 2 and 4 compared with the amounts originally present in the sputum solutions. In run 3, the initial protein concentration was about 20% of that for the sputa used for runs, 1, 2 and 4. The combination of a relatively large weight of chrysotile (1g) and low sputum total protein concentration (200 mg.%) in run 3 resulted in incomplete protein uptake during the 35 minutes of the test. It would

have been informative to have continued this run beyond 35 minutes. Unfortunately, the swelling of the chrysotile caused difficulties in synchronisation of the sputum and buffer flow rates.

The technical problems encountered in both methods prevented the assessment of protein adsorption from sputum onto chrysotile over long periods. However, it is clear from both approaches that, when chrysotile is placed in sputum solution, the sequence of events is an initial uptake of water followed by more gradual uptake of protein and that the system probably reaches the steady state in about 30 minutes. Published work on protein adsorption onto chrysotile relates to models using single proteins. Blount, Holt and Leach (1966) found that the maximum uptake of bovine serum albumin had occurred after 24 hours incubation at 37^{o}C while Morgan (1974) reported that maximum uptake of human serum albumin onto chrysotile required 15 minutes at 25^{o}C and that there was 'little change in the amount of albumin adsorbed after the first one or two minutes.'

The time elapsing between chrysotile deposition on the human airway mucosa and its arrival in the lung parenchyma is not known therefore the period that retained chrysotile may remain immersed in airway secretion is conjectural. After consideration, an incubation time of 18 hours was chosen for the protein adsorption investigations that follow. This interval corresponds with the overnight rest period for exposed workers.

5.4 Analysis of Protein Uptake by Chrysotile from Sputum

5.4.1 Materials and Method

Sputa were selected that spanned the range 620-2100 mg.% total protein. Each sputum was prepared as described in Section 2.1.4 and then divided into one volume (2ml) for adsorption and the remainder for use as a control. 20mg sieved chrysotile was added to 2ml of the sputum solutions and the resultant suspensions were incubated at 37°C for 18 hours. The sputum solutions were then centrifuged at 14,000g for 6 minutes and the supernatant fluids subjected to total protein assay, electrophoretic separation and immunoanalysis as described in Chapter 2. The protein taken up by the chrysotile was calculated by difference between the untreated control solution and the chrysotile-treated solution for each specimen.

5.4.2 Results

Uptake of total protein ranged between 37± 11.1 and 54± 5.3 mg/g chrysotile and was independent of the total protein concentration in the sputum. An overall mean uptake of 44.8± 7.7 mg protein/g chrysotile was obtained.

Electrophoresis showed that part of each fraction was taken up independent of the starting concentration of particular fractions. The mean weight of the beta/gamma fraction was approximately equal to the combined weights of the other three fractions (beta/gamma 23± 3.6; albumin 6.8± 3.7; alpha globulin 7.3± 3.3 and lysozyme 7.5± 3.3 all mg/g chrysotile).

Table 20 Protein Uptake by Chrysotile from Sputum

Sputum protein fraction	concn. mg.%	Uptake mg.protein/g.chrysotile Mean ± s.d.
Sputum specimen 1		
albumin	87	7 ± 0.7
alpha globulin	124	11 0.4
beta/gamma globulin	322	26 2.9
lysozyme	87	8 1.2
total protein	620	54 5.3
Sputum specimen 2		
albumin	133	2 1.2
alpha globulin	159	8 1.4
beta/gamma globulin	572	25 4.5
lysozyme	76	5 2.5
total protein	940	40 6.6
Sputum specimen 3		
albumin	220	7 4.0
alpha globulin	207	7 3.0
beta/gamma	701	23 6.0
lysozyme	172	12 4.2
total protein	1300	48 6.6
Sputum specimen 4		
albumin	630	11 6.7
alpha globulin	292	3 5.6
beta/gamma globulin	1094	18 4.1
lysozyme	84	5 1.3
total protein	2100	37 11.1

Table 20 cont

Mean values - all four specimens combined			
albumin	87-630	6.8	3.7
alpha globulin	124-292	7.3	3.3
beta/gamma globulin	322-1094	23	3.6
lysozyme	87-172	7.5	3.3
total protein	620-2100	44.8	7.7

Table 21 Uptake of PAS-Staining Glycoprotein by Chrysotile from Sputum

Sputum specimen 1	concn. mg.%	Mean	± s.d.
$alpha_1$	54	4	± 0.6
$alpha_2$	72	6	0.8
beta/gamma	320	27	2.5
total	446	37	1.3
Sputum specimen 2			
$alpha_1$	73	5	0.9
$alpha_2$	110	5	1.3
beta/gamma	548	21	7.5
total	731	31	3.2
Sputum specimen 3			
$alpha_1$	272	8	3.5
$alpha_2$	282	12	3.0
beta/gamma	354	10	5.0
total	903	30	3.8
Sputum specimen 4			
$alpha_1$	305	9	5.9
$alpha_2$	333	7	3.3
beta/gamma	748	6	5.0
total	1386	22	4.7
	Mean values - all four specimens combined		
$alpha_1$	54-305	6.5	2.4
$alpha_2$	72-333	7.5	3.1
beta/gamma	320-748	16	9.7
total	446-1386	30	6.2

Table 22 The Effect of Immunoprecipitation in Sputum Solution after Protein Adsorption by Chrysotile

Sputum total protein mg%	620		940		1300		2100	
	C	T	C	T	C	T	C	T
Specific protein								
prealbumin	3.0	2.6	1.0	2.0	1.5	2.1	3.0	2.0
albumin	6.0	6.8	2.5	2.8	2.5	3.2	2.0	2.3
haptoglobulin	6.5	4.5	5.5	3.7	4.0	1.8	0	0
ceruloplasmin	5.0	2.3	2.0	1.9	2.0	2.3	2.0	0.4
$alpha_2$ macroglobuline	3.0	3.1	2.0	3.1	0	0	0	0
transferrin	2.0	2.3	2.0	1.0	1.0	1.7	3.0	3.2
$beta_1 C beta_1 A$	3.0	4.0	4.5	4.5	3.0	3.0	2.0	2.5
gamma a	4.5	4.0	3.6	3.0	2.5	2.5	3.5	2.4
gamma g	5.0	3.0	2.0	1.0	2.0	1.3	5.0	4.3
gamma m	4.0	3.7	4.5	4.6	3.0	3.7	2.0	0.8
gamma e	0	0	0	0	6.0	2.8	5.0	7.0
$alpha_1$ acid glycoprotein	2.0	1.8	1.0	1.3	3.5	3.2	4.0	3.1
$alpha_2$ HS glycoprotein	3.0	3.1	3.0	2.5	3.0	2.8	2.0	0.5
$alpha_1$ lipoprotein	0	0	0	0	2.0	2.1	3.5	3.0
beta lipoprotein	3.0	3.4	4.0	3.3	3.5	2.4	4.5	2.0

C = untreated sputum solution

T = sputum solution after chrysotile adsorption

All values given as diameter of immunoprecipitate (mm).

Two thirds of all protein taken up was PAS reacting glycoprotein. The mean weight of beta/gamma PAS fraction taken up was approximately equal to the combined weights of the $alpha_1$ and $alpha_2$ PAS fractions (beta/gamma 16± 9.7; $alpha_1$ 6.5± 2.4; $alpha_2$ 7.5± 3.1 all mg./g. chrysotile). These results are shown in Tables 20 and 21.

The ratio of the mean weights of protein fractions taken up by chrysotile is approximately 1:1:3:1 (albumin:alpha globulin:beta/gamma globulin:lysozyme). For PAS reacting glycoprotein the mean weight ratio is 1:1:2, ($alpha_1$:$alpha_2$:beta/gamma).

An attempt was made to identify fifteen proteins by immunodiffusion using specific antisera. None of these proteins was totally adsorbed by the chrysotile. In some analyses the diameter of the immunoprecipitate obtained after adsorption exceeded the diamter of the immunoprecipitate obtained from the untreated, control sputum. These results are shown in Table 22.

5.4.3 Discussion

The mean value obtained for uptake of total protein, 44.8± 7.7 mg. protein/g. chrysotile (surface area 21.7m^2/g. chrysotile) approximates to the finding of 45mg. human albumin/g. chrysotile (surface area 22.4m^2/g. chrysotile) obtained by Morgan (1974). Blount, Holt and Leach (1966) and Morgan (1974) consider that the coverage resulting from an uptake of 45mg. albumin/g. chrysotile constitutes a monolayer of albumin onto the exposed surface of the chrysotile. The current study differs from the single protein systems used in both previous investigations

since sputum is a multiple protein system and the results show that a proportion of each protein was taken up by the chrysotile. If the available surface area of chrysotile is the primary determinant of the weight of protein adsorbed, the results from the current study suggest that large protein molecules such as gamma globulins (M.Wt. 160-190,000) effectively occlude areas of the chrysotile surface that would otherwise be covered by smaller molecules such as albumin (M.Wt. 66,300) roughly in proportion to their monomeric molecular weights. This poses the question whether such a coat can produce a coherent film of protein.

PAS reacting glycoprotein contributes two thirds of all protein taken up and this explains why chrysotile coated with sputum proteins stains strongly by the PAS method.

The immunodiffusion results reveal a paradox since some tests produced immunoprecipitates of greater diameter from sputum exposed to chrysotile than those obtained with the same antiserum against the untreated sputum solution. These results are shown in Table 22: the results for albumin are especially noteworthy since the uptake of albumin is confirmed by the electrophoresis results (see Table 21). A possible explanation for this is that chrysotile preferentially takes up dimeric albumin thus allowing the monomer to diffuse more rapidly during the test period. Because of this anomoly, the immunodiffusion results cannot be interpreted quantitatively; however, it can be seen that no protein that is present in untreated sputum was totally removed by exposure to chrysotile.

The results from cation exchange analysis of sputum (see Section 2.5.1.3) showed a trend toward loss of a beta/gamma protein fraction eluting at 0.23/0.30 molar on the sodium chloride gradient. An attempt was therefore made to monitor the uptake of this fraction in sputum containing sieved chrysotile.

5.5 Protein Uptake by Chrysotile from Sputum as Shown by Cation Exchange Separation

5.5.1 Materials and Method

Sputum from an unexposed donor was prepared as described in Section 2.1.4. 0.1ml of the solution was taken for cation exchange separation as described in Section 2.5.1. 20mg. sieved chrysotile was added to 2 ml. of the sputum solution and incubated at 37^{o}C in a capped 2ml. polystyrene container. The container was shaken to mix at intervals and 0.2ml. suspension was transferred to a capped polythene centrifuge tube and centrifuged at 14,000 g for six minutes. 0.1ml of the resulting supernatant was taken for cation exchange separation as previously described. The area enclosed by each peak obtained during elution was integrated and expressed as a percentage of the area of the corresponding peak obtained from fractionation of the control solution. All fractions were dialysed against PVP as described in Section 2.5.1 and subjected to electrophoresis as described in Section 2.3.1. Samples from each fraction were spotted onto cellulose acetate strips and stained for PAS reacting protein.

Table 23 Protein uptake by chrysotile from sputum as shown by cation exchange fractionation on CM sephadex C50

	Fraction eluted with starting buffer			Fraction eluted on the sodium chloride gradient (0.0 to 1.0 Molar)		
	1	2	3	0.23	0.37	0.74
Sampling time						
7 minutes	0	25	32	80	95	85
15 minutes	0	20	13	22	77	87
30 minutes	0	20	31	70	96	92
2 hours	0	17	28	72	97	100
4 hours	0	17	42	72	97	100
	% protein taken up by chrysotile from each peak					
% mean uptake	0	19.8	29.2	63.2	92.4	92.8
± s.d.	-	3.3	10.5	23.4	8.7	7.1

Electrophoretic Mobility (control solution)	albumin alpha beta gamma	beta/ gamma	$beta_3$	$beta_3$/ gamma	gamma	gamma
PAS reaction	+	+	+	+	+	

5.5.2 Results

The trace obtained from the control solution was similar to that shown in Figure 16 except that three peaks occurred on the sodium chloride gradient. These peaks were seen at 0.23; 0.37 and 0.74 Molar. During the incubation period the test suspension was sampled at 7; 15; and 30 minutes and at 2 and 4 hours.

No protein was lost from the first peak eluting with the starting buffer but part of the second and third peaks eluting with starting buffer were taken up by chrysotile. The results obtained for losses sustained by these two fractions, however, are not consistent with the sampling sequence (see Table 23). Similar inconsistencies occur in the percentage losses to chrysotile of the gradient fractions eluting at 0.23 and 0.37 Molar. The fraction eluting at 0.74 Molar shows a progressive loss to chrysotile that is completed within two hours.

The electrophoretic mobility of the fractions conformed with the findings obtained from cation exchange analysis of the sputum reported in Section 2.5 (Table 15).

All adsorbed fractions stained positively for PAS reacting glycoprotein.

5.5.3 Discussion

The findings indicate that sieved chrysotile exhibits preferential adsorption of proteins from sputum. It can be seen from Table 23 that the cation exchange method is either insufficiently sensitive to yield accurate information on the rate at which a particular protein fraction was taken up by chrysotile or interchange between the various fractions

occurred during the test period - proteins from one fraction displacing others all ready adsorbed. It is probable, however, that after two hours, irreversible adsorption of the entire 0.74 Molar fraction had taken place. This fraction is a gamma globulin and a second gamma globulin fraction eluted at 0.37 Molar was almost wholly taken up after four hours.

The $beta_3$ fraction was partly taken up both as a single component (fraction 3, starting buffer) and also when combined with gamma globulin (0.23 Molar fraction). It would have reinforced the observation made earlier in Section 2.5.1.3 - that loss of the 0.23 Molar fraction may be associated with progressive to fibrosis - if chrysotile had shown higher avidity for this protein fraction but the investigation indicates that gamma globulins predominate the protein coat when chrysotile is immersed in sputum.

At this point it was decided to examine the effect on human somatic cells of exposure to chrysotile that had been coated with these sputum proteins.

CHAPTER SIX

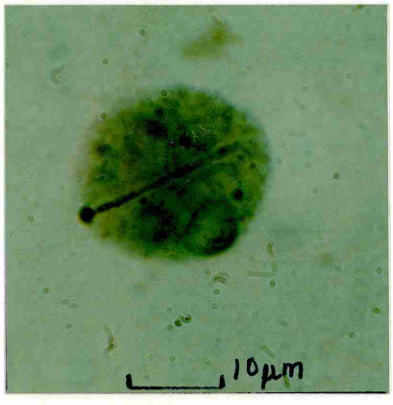
The Effect of Chrysotile on Human Somatic Cells

The Effect of Chrysotile on Human Somatic Cells

6.1 Introduction

In 1964, House reported that cell culture medium, sterilised by filtration through asbestos filters, exhibited toxicity towards hamster fibroblasts. He found that the first portion of culture medium passing through the filter was the most toxic fraction and that later portions showed sequentially less toxicity towards the cells. MacNab and Harington (1967), in a later study on the haemolytic property of various mineral dusts, found that chrysotile fibres lysed sheep erythrocytes suspended in isotonic saline. This finding was confirmed by Harington, Miller and MacNab (1971) who found that chrysotile also lysed human erythrocytes. These workers suggested a tentative association between the haemolytic property and cytotoxicity of chrysotile. Subsequently, Desai, Hext and Richards (1975) found that the haemolytic property of chrysotile was inhibited by, lysed rabbit erythrocytes, bovine serum and also ox pulmonary surfactant. They concluded that the 'binding of relatively non-soluble serum and surfactant materials to asbestos dust (particularly chrysotile) is important to prevent haemolysis.' The protective action of foetal calf serum for cells in the presence of chrysotile was shown by Allison (1973) who found that mouse peritoneal macrophages that were attached to chrysotile fibre and suspended in 10% foetal calf serum, remained healthy for at least a week. In the current study, intact histiocytes containing endocytosed chrysotile fibre and carbon particles in the same cell were found in sputa from asbestotic donors (see Figure 27). This

Figure 27 Lung histiocyte containing endocytosed chrysotile fibre and also carbon particles



observation indicates that lung histiocytes can endocytose chrysotile fibre at the epithelial surface without incurring immediate cell death.

It would be informative to reproduce this stage *in vitro* to investigate endocytosis of chrysotile by histiocytes alone and by histiocytes containing carbon but it is difficult to isolate human lung histiocytes. All current separation techniques result in admixture with other somatic cells and also with micro-organisms that are present as commensals in the upper airway. A preliminary investigation was made to establish whether uncoated chrysotile is cytolytic toward human somatic cells.

6.1.1 Materials and Method

Heparin (Pularin; Duncan Flockhart and Co. Ltd.)

T.C. 199 culture medium single strength (Wellcome Research Labs.)

Sieved chrysotile (for preparation see Section 1.2.5).

Ten ml venous blood fromaa normal donor was added to a polystyrene tube containing 200 i.u. heparin. After gently mixing the tube was centrifuged at 600g for 15 minutes and the supernatant plasma removed. The leucocyte layer was pipetted into 4 ml T.C. 199 solution and mixed by gentle shaking of the tube. The suspension was centrifuged at 400g for 10 minutes and the resulting cell deposit was given two further washes of 4ml T.C. 199 solution. The washed leucocytes were then suspended in 2ml. T.C. 199 solution.

A suspension of 40mg sieved chrysotile in 4ml T.C. 199 solution was prepared by mixing on a roller mixer (Denley Spiramix 5, Denley-Tech. Ltd. Sussex) for 10 minutes.

Polystyrene tubes were set up containing:

	Control	Test
Leucocyte suspension	1 ml	1 ml
T.C. 199 solution	1 ml	Nil
Chrysotile suspension	Nil	1 ml

The tubes were placed on a roller mixer and incubated at 37°C. Counts were made on the cell suspensions at the start and at 30 minute intervals for the first two hours and also at 18 hours. 20 mm^3 of mixed cell suspension was added to 0.5ml white cell diluting fluid. After mixing the suspension was loaded into an improved Neubauer haemacytometer (depth 0.1mm) and cell counts made over 8 mm^2.

After 18 hours at 37°C the cell suspensions were centrifuged at 400mg for 10 minutes and smears made from the centrifuge deposit. The smears were fixed and stained by the Papanicoloau and Jenner Geimsa methods as described in Section 3.1.2.

6.1.2 Results

The cell counts on the test suspensions showed that after exposure to uncoated sieved chrysotile for one hour there were no cells surviving. By contrast the cell density in the control suspensions was not significantly different

after 18 hours, p = 0.05. Examination of the stained smears showed that the cells in the control suspensions had undergone no gross morphological change after 18 hours.

Cell Counts are given in Table 25.

6.1.3 Discussion

The culture medium, T.C. 199 Wellcome is a modified Earle's Balanced Salt solution (Morgan, Morton and Parker, 1950) containing twenty amino acids but no protein constituent. It can be seen from the 'Test' results that uncoated chrysotile is cytolytic toward nucleated human blood cells and that no protection is afforded the cells by the presence of amino acids. It is probable that the cytotoxic agent reported by House (1964) was chrysotile fibre washed from the asbestos filter pads into the culture medium subsequently used to grow the hamster fibroblasts. When the free chrysotile dust had been washed out of the filter, the resulting filtrate was no longer toxic to the cells.

The extension of the current investigation into the response by histiocytes to chrysotile is hampered by technical difficulties; the procurement of living human alveolar histiocytes presents a major obstacle. Only occasional sputum specimens contain living histiocytes but, when present, these cells have subsequently remained alive for 5 to 11 days at $+5^{\circ}C$ as judged by the Trypan Blue exclusion test. Similarly, post mortem lungs only occasionally yield living histiocytes. Cell fractions from both sputum and lung tissue was invariably contaminated with micro-organisms and these small cells cannot

Table 25 The effect of sieved chrysotile on nucleated blood cells.

Cell Counts - Total Nucleated Cells/mm.

Control series						
Mean	1579	1500	1219	1438	1094	1156
± s.d.	176	467	388	161	258	425
Test series						
Mean	1579	188	0	0	0	0
± s.d.	176	125	-	-	-	-
Time (hr)	0	½	1	1½	2	18

Controlseries - Comparison of the means at 0 and 18 hours

$$t_{(calc)} = 2.19 \quad t_{(table)} = 2.23 \quad p = 0.05$$

be totally separated from somatic cells by present methods. It is also difficult to fractionate cells in sputum. The first requirement is to reduce mucus viscosity. Investigation showed that it cannot be achieved with the ultrasonic probe without disintegrating most of the cells in the mucus. Most mucolytic agents are cytotoxic to histiocytes although one such reagent that is not is dithiothreitol (Shah and Dye, 1966) but mucolysis is not permanent and is not even temporarily achieved in some sputum specimens. An alternative approach investigated was to disperse sputum in a large volume of isotonic saline using an homogeniser. It was possible to work with the resultant cell suspensions but, despite many washings in phosphate buffered saline, the cells agglutinated during overnight storage at +5^{o}C. During this investigation it was found that a significant proportion of histiocytes adhered to the glass walls of the centrifuge tube just below the interface of the density gradient cushion. Phase contrast microscopy of these cells showed no Brownian movement of organelles in the cytosol and Trypan Blue passed quickly through the cell membranes showing that the cells were dead.

Because of these difficulties it was decided to examine the effect of exposing blood monocytes to sieved chrysotile, both uncoated and coated with sputum proteins. A firm conclusion that blood monocytes were the precursors of tissue histiocytes was drawn in a major publication (Van Furth, Cohn, Hirsch, Humphrey, Spector and Langevoort, 1972) and this is now generally accepted by cell biologists - see for example Carr, Hancock and Ward (1977) and Moore (1978).

The newly formed monocyte leaves the bone marrow soon after production and passes to the tissues by way of the peripheral blood. According to Goldman (1972) the total number of histiocytes exceeds the number of circulating monocytes by a factor of 400 but although the lifespan of the histiocyte is not established, its duration is months rather than days (Goldman, 1972). The monocyte is now used as a substitute for the tissue histiocyte by cell biologists (Ackerman, Tuckerman and Douglas, 1981), immunologists (Rolan Pleszczynski and Churchill, 1978) encologists (Currie and Hadley, 1977) and clinical pathologists (Dent and Cole, 1981). All the foregoing workers used a one-step density gradient method employing a mixture of Ficoll (Pharmacia Ltd.) and sodium diatrizoate either according to the original technique of Boyum (1966) or commercial formulations of the mixture based on Boyum's work.

6.2 The effect of uncoated sieved chrysotile and chrysotile coated with sputum proteins on the mononuclear cell fraction of human blood

6.2.1 Preparation of sieved chrysotile coated with sputum proteins

6.2.2 Method

Homogenised sputum was prepared as described in Section 2.1.4. 1ml treated sputum was added to 20mg sieved chrysotile in a polystyrene container and mixed by placing on a roller mixer for 15 minutes at room temperature. The resultant

suspension was incubated at 37°C for two hours and then centrifuged at 14,000g for 6 minutes. The supernatant sputum was discarded and the chrysotile deposit washed free from unadsorbed protein by mixing with 2ml deionised water and centrifuging at 14,000g for 6 minutes. After two further washes the chrysotile was resuspended in 2ml T.C. 199 culture medium.

During incubation the chrysotile had swollen and formed a sediment 8mm deep in the tube. This sediment compacted to 2mm after centrifuging. It was noticed that the supernatant sputum was markedly clearer than an untreated sample of the same sputum. Microscopy using crossed Nicol prisms showed that treated chrysotile retained the refringent property of untreated chrysotile and chrysotile fibre as seen in exposed sputum.

15ml venous blood from a normal donor was added to a polystyrene tube containing 300 i.u. heparin and gently mixed. 20ml isotonic phosphate buffered saline was added and the suspension was layered onto 4ml Ficollpaque (Pharmacia Ltd.) in a 1 oz. polystyrene universal container. The container was centrifuged at 400g for 30 minutes and the interface deposit was pipetted into 2ml T.C. 199 solution. The cells were gently suspended by tapping the tube and then centrifuged at 400g for 20 minutes. After two further washes the cells were resuspended in 2ml T.C. 199 solution. One drop of cell suspension was used to prepare smears that were fixed and stained by Papanicoloau and Jenner Geimsa methods as described in Section 3.1.2. Cell counts were made on the cell suspension as described in Section 6.1.1. Coated sieved chrysotile was prepared as previously described using sputum

from an asbestotic donor. 1ml of the chrysotile suspension (= 10mg coated chrysotile) was placed in a polystyrene tube, centrifuged at 2,000g for 10 minutes and the supernatant discarded. This tube was labelled 'coated chrysotile'. Uncoated sieved chrysotile was prepared as described in Section 6.1.1. 1ml of the suspension (=10mg uncoated, sieved chrysotile) was placed in a second polystyrene tube, centrifuged at 2,000g for 10 minutes and the supernatant discarded. This tube was labelled 'uncoated chrysotile'. 0.5ml of cell suspension was added to each of the tubes and also to a third clean polystyrene tube labelled 'Control'. All three tubes were incubated at 37°C. After 1 hour, 4 hours and 22 hours incubation the tubes were shaken to resuspend the cells and samples taken for cell counts as previously described. At 22 hours the residual cell suspension in all tubes was used to prepare smears that were fixed and stained as previously described.

6.1.3 Results

Counts on the cell suspensions containing uncoated chrysotile showed that most of the cells (about 98%) were lost after one hour exposure.

After 4 hours incubation a decline in cell density occurred in both the control cell suspension and the cell suspension containing coated chrysotile when compared with the starting cell density. There was no significant difference in cell content between the two preparations at 4 hours, $p = 0.05$.

At 22 hours the cell content of both the control and test suspension containing coated chrysotile showed a further decline and this cell loss was significantly greater in the test suspension containing coated chrysotile than the control cell suspension, $p = 0.05$.

Microscopic examination of the stained preparations made after 22 hours incubation of the cells showed that both the control and the test suspension contained cells with irregularly shaped nuclei with pale-staining chromatin of uniform texture. Some of the cells had no visible cytoplasm.

6.2.4 Discussion

The results show that cells suspended in T.C. 199 culture medium are afforded protection against the cytotoxicity of chrysotile for at least four hours if the chrysotile is coated with sputum proteins prior to use but significantly fewer cells survive after 22 hours exposure, see Table 26.

In contrast to the unfractionated cell investigation, the control suspension showed a decline in population density after 22 hours, see Table 26. The cell morphology in the smears taken at 22 hours suggest that this is due to increased fragility of some cells probably as a consequence of exposure to the reagent used for the one-step gradient fractionation. The appearance of the damaged cells was similar to that described by Whitby and Britton (1953) as degenerate lymphocytes. An investigation was made to try to establish the cell type giving rise to these cells.

Table 26 The effect of treated and untreated chrysotile on the fractionated mononuclear cells from venous blood

Cell counts - Cells/0.1 mm^3

Time (hr)	Control		Coated chrysotile		Uncoated chrysotile	
	Mean	±s.d.	Mean	±s.d.	Mean	± s.d.
0	25.7	3.44	25.7	3.44	25.7	3.44
1	25.1	3.08	25.8	1.42	1.70	0.44
4	18.1	2.07	17.7	1.50	0.75	0.13
22	18.8	1.19	15.0	1.35	0	-

Comparison of the means - $t_{(calc)}$

	Control	Coated chrysotile	Control vs coated
0 and 1 hr	0.33	0.12	0.51
0 and 4 hr.	5.31	6.02 At 4 hr.	0.51
0 and 22 hr.	5.33	8.11 At 22 hr.	5.86

n = 8 $t_{(table)}$ = 2.14 p = 0.05

6.3 To Identify the Cell Type Producing the 'Smear Cell' After Ficollpaque Fractionation

6.3.1 Method

Thirty six ml venous blood from a normal donor was added to each of two polystyrene tubes containing 350 i.u. heparin. The tubes were centrifuged at 2,8000g for 20 minutes and the resulting leucocyte layers pipetted into a tube containing 10 ml phosphate buffered saline. The cell suspension was layered onto a cushion of 5 ml. Ficollpaque (Pharmacia Ltd.) and centrifuged at 400g for 20 minutes. The interface fraction was transferred to a tube containing 5ml T.C. 199 solution and, after mixing, centrifuged at 400g for 20 minutes. The cell deposit was given two further washings in 5ml. T.C. 199 solution and then suspended in 10ml T.C. 199 solution.

A cell count was carried out and Jenner Geimsa - stained smears prepared using sputum from a) an asbestotic donor, and b) an unexposed donor as described in Section 6.2.2. A tube containing uncoated sieved chrysotile was prepared as described in Section 6.1.1.

2ml cell suspension was added to each chrysotile tube and also to a clean tube as a Control. The tubes were incubated at $37^{o}C$ and samples taken at intervals for cell counts and stained smears using the previously described methods. Cell differential counts on the stained films were obtained by classifying 500 cells in each smear and calculating the percentage proportion of each cell type. These percentages were used to calculate the absolute number of each cell type in unit volume of suspension (0.1 mm^3).

Table 27 The Effect of Treated and Untreated Chrysotile on the Fractionated Mononuclear Cells from Venous Blood

Cell Counts - Cells/0.1 mm^3

Time (hr)	Control		Coated chrysotile (asbestotic)		Coated chrysotile (unexposed)		Uncoated chrysotile	
	Mean	±s.d.	Mean	±s.d.	Mean	±s.d.	Mean	±s.d.
0	28.0	3.1	28.0	3.1	28.0	3.1	28.0	3.1
1	25.1	3.0	25.5	3.0	27.2	1.9	0	
18	29.0	2.1	17.1	2.4	22.4	3.9	0	
25	23.9	4.6	17.9	3.3	15.6	4.5	0	

% Differentials - Neutrophils/lymphocytes/monocytes/smear cells

0	5/56/39/0	5/56/39/0	5/56/39/0	5/56/39/0
1	6/40342/12	11/49/16/24	10/37/43/10	-
18	5/48/35/12	6/37/11/46	3/37/7/53	-
25	3/46/3/48	5/66/2/10	11/78/3/8	-

Table 28 The Effect of Treated and Untreated Chrysotile on the Fractionated Mononclear Cells from Venous Blood

Comparison of the means $t_{(calc)}$

	Control	Coated chrysotile (asbestotic)	Coated chrysotile (unexposed)	Uncoated chrysotile
0 vs 1 hr.	1.90	1.64	0.62	-
0 vs 18 hr.	0.76	6.81	3.18	-
0 vs 25 hr.	1.96	6.31	6.42	-

at 25 hr. coated (asbestotic) vs coated (unexposed) 1.17

$t_{(table)} = 2.14$ $p = 0.05$

Table 29 The Absolute Numbers of Each Cell Type in Unit Volume (0.1 mm^3) of Cell Suspension

	At start	After 1 hour at 37°C		
		Control	Chrysotile (asbestotic)	Chrysotile (unexposed)
Neutrophils	1.4	0.3	2.81	2.72
Lymphocytes	15.7	10.04	12.50	10.06
Monocytes	10.9	10.54	4.08	11.70
Smear cells	0	3.01	6.12	2.72
		After 18 hours at 37°C		
Neutrophils	1.4	1.45	1.03	0.67
Lymphocytes	15.7	13.92	6.33	8.29
Monocytes	10.9	10.15	1.88	1.57
Smear cells	0	3.48	7.87	11.87
		After 25 hours at 37°C		
Neutrophils	1.4	0.72	0.90	1.72
Lymphocytes	15.7	10.99	11.81	12.17
Monocytes	10.9	0.72	0.36	0.47
Smear cells	0	11.73	1.79	1.25

6.3.2 Results

After 25 hours incubation at 37°C, the control suspensions showed no significant loss of cells, $p = 0.05$. The cell suspensions containing coated chrysotile showed no significant cell loss at 1 hour but significant cell losses did occur in these cell suspensions after 25 hours at 37°C. The cell densities in the tubes containing chrysotile (asbestotic) and chrysotile (unexposed) were not significantly different at 25 hours. The cell suspensions containing uncoated chrysotile had undergone complete cytolysis after 1 hour at 37°C.

When the absolute number of each cell type in unit volume of cell suspension is compared before and after the 25 hour incubation period, it can be seen that changes occurred in cell distribution, see Table 29. The loss of lymphocytes was $4.04 \pm 0.60/0.1\ mm^3$ (25.73%) and the loss of monocytes was $10.38 \pm 0.18/0.1\ mm^3$ (95.25%).

Smear cells had appeared in both the control and the cell suspensions containing coated chrysotile at one hour. The number of these cells increased progressively in the control suspension up to 25 hours. Both suspensions containing coated chrysotile showed a more rapid increase in smear cells than the control up to 18 hours but many of the smear cells had disappeared after 25 hours, -77% in Chrysotile (asbestotic) and -89% in chrysotile (unexposed).

6.3.3 Discussion

Using the cell fractionation treatment described, the

smear cells derive from the blood monocytes. The fragility of these cells is illustrated by their subsequent loss in the coated chrysotile cell suspensions.

Most workers who use the monocyte as a cell model, resuspend the interface fraction in cell culture medium containing 10% serum - usually autologous serum or foetal calf serum. The monocytes attach to the walls of the container and the free mononuclear cells can be washed away. The adherent monocytes are then detached from the surface by a mild proteolytic agent and then subjected to experiment. The aim in the present work however, was to study the interaction between monocytes and coated and uncoated chrysotile in the absence of other proteins. This lack of serum in the culture medium may have contributed to the low monocyte survival rate (5%) after 25 hours at 37^{o}C since the yield from serum-containing culture medium is higher than 5%. Currie and Hedley (1977) report yields of 12 to 15% and Rola-Pleszczynski and Churchill (1978) had recoveries of 22.4% while Ackermann, Zuckermann and Douglas (1981) have published results that indicate almost total recovery of blood monocytes after fractionation. The results from the current investigation suggest that monocytes cannot develop in vitro in the absence of serum proteins and consequently cannot be used for this study without modifying the experimental system to take this into account.

Further work on the technique was halted because a source of human alveolar histiocytes became available with the introduction of fibreoptic bronchoscopy, see Section 1.2.3.5, to aid the diagnosis of chest diseases at St. Luke's hospital.

The routine procedure as practised here, is to irrigate matching lung fields in both lungs of an individual with a lesion present in only one lung. For a particular patient, the cytological findings obtained from the washings from the unaffected lung are used as control data when interpreting the cytology of the affected lung and also to compare the results with those obtained from normal individuals. Toward the end of this phase of the chrysotile study, the cell content of bronchial washings from two apparently normal individuals was found to consist almost enitrely of histiocytes. The opportunity was thus presented to examine the effect of sputum coated and uncoated chrysotile on suspension of these histiocytes.

6.4 The Effect of Coated and Uncoated Chrysotile on Human Alveolar Histiocytes.

6.4.1 Material and Method

Bronchial washings in 1 oz. polystyrene universal containers were centrifuged at 600g for 10 minutes and the resulting cell deposit was resuspended in 10ml. T.C. 199 culture medium. Cell counts on these suspensions were carried out as described in Section 6.1.1. Cell viability was assessed using the Trypan Blue exclusion test (Whitaker, 1975) by counting 500 cells at x600 magnification and classifying each as stained or unstained (non-viable or viable). Smears were prepared and stained as described in Section 3.1.2 with Jemma Geimsa stain.

Polystyrene tubes were prepared containing in one series 10mg uncoated chrysotile and in another series 10mg coated chrysotile (unexposed donor) as described in Section 6.2. 2ml cell suspension was added to the tubes and also to a control tube which were then incubated at 37°C for 18 hours. Each cell suspension was then mixed and the cell count, viability count and stained smears were repeated.

6.6.2 Results

The control series showed a mean cell loss of about 34% (35.8 and 32%) after incubation and the loss of viable cells during this time was about 55% (56.0 and 54.5%).

The mean cell loss in the coated chrysotile suspensions was 51.5% (49.54 and 53.37%) and the mean loss of viable cells during the incubation period was about 72% (74.0 and 70.29%).

No cells survived the incubation period in suspensions containing uncoated chrysotile.

The histiocytes in the stained smears prepared from the control and the coated chrysotile suspensions after incubation showed no gross morphological change.

6.4.3 Discussion

The results indicate that not all alveolar histiocytes are viable when harvested by broncho-alveolar lavage. This is supported by the results of viability counts on histiocytes from a series of bronchoscopies (n=8) in which the mean histiocyte viability count was 56.88 $\pm$ 23.02%. Also, the

histiocyte viability count for two patients with asbestosis was 80% and 75%.

Although the investigation is too limited for statistical comment, the results suggest that, in the presence of chrysotile coated with sputum proteins and in the absence of other proteins, histiocyte viability is impaired and fragility is increased. The cytolytic property of uncoated chrysotile is unequivocal.

The results also suggest that broncho-alveolar lavage offers a means of obtaining human lung histiocytes provided that these cells can be fractionated from the general cell content of the wash. A method that might achieve this has been suggested by Professor T.G. Baker, University of Bradford, and the preliminary findings indicate that this may be possible. Pronase is used as the mucolytic agent and the mixed cell population of the wash fractionated by velocity sedimentation through a continuous gradient formed from bovine serum albumin.

6.5 The Cell Fractionation of Bronchial Washings by Velocity Sedimentation

6.5.1 Introduction

In 1969 Miller and Phillips showed that a mixed population of living somatic cells can be partitioned into its constituent cell types by utilising the physical properties of cell size and cell density.

Table 30 The Effect of Coated and Uncoated Chrysotile on Human Alveolar Histiocytes from Two Donors

Time (hr)		Control		Coated chrysotile		Uncoated chrysotile	
	Cells/0.1mm^3	a	b	a	b	a	b
	Mean	10.9	25.2	10.9	25.2	10.9	25.2
Start	s.d.	0.83	5.92	0.83	5.92	0.83	5.92
	Viability	73	89	73	89	73	89
	Cells/0.1mm^3						
	Mean	7.0	17	5.5	11.75	0	0
18	s.d.	1.2	0.82	0.93	0.50	-	-
	Viability	50	60	43	50		
	Comparison of the means ($t_{(calc)}$)						
0vs18		7.5	5.19	12.0	5.77		
p=0.05 $t_{(table)}$ =		2.14	2.11	2.14	2.11		
Control vs coated		a9.05	b3.13				
p=0.05 $t_{(table)}$ =		2.45	2.14				

A density gradient is established in a column of phosphate buffered saline by adding bovine serum albumin the concentration of which increases progressively with column depth. When a heterogenous suspension of cells is loaded onto the top of the column, previously cooled to reduce convection currents, the cells sediment under gravity at a rate dependent upon the size and density of the cells. If the column of saline is carefully drained from below after a suitable interval, the first fraction will contain the smallest, most dense cells and the last cell fraction will contain the largest, lightest cells.

6.5.2 Materials and Method

Pronase (Light and Co. Ltd.)

Phosphate buffered saline (Flow Labs. Ltd. Irvine)

Bovine serum albumin (Armour Ltd. Eastbourne)

Bronchial washings

'Staput' velocity sedimentation cell separator S.P. 120 (Johns Scientific, Toronto)

Gradient maker apparatus (Pharmacia Ltd.)

Bronchial washings were collected as described in Section 1.2.3.5 and processed as described in Section 3.2.1 until the last centrifuging stage. At this step, the resulting cell deposit was resuspended in 10 ml phosphate buffered saline (PBS). 250ml of 0.3% bovine serum albumin (BSA) in PBS and 250ml of 2.0% BSA in PBS were prepared. The cell suspension and the BSA solutions were cooled to $+3^{\circ}C$ in a refrigerator. The 'Staput' apparatus was assembled

as shown in Figure 28 on a tray containing water and melting ice. The dead space in the tubing between the gradient maker and the sedimentation chamber as far as the baffle, was filled with PBS and the apparatus shrouded in polythene film and allowed to stand for two hours before use.

250ml 0.3% BSA in PBS solution was loaded into reservoir A of the gradient maker and 250ml 2.0% BSA in PBS solution was loaded into reservoir B of the gradient maker. The cell suspension was carefully layered into the sedimentation chamber onto the solution above the baffle D using a disposable pipette. The gradient maker was switched on and the BSA gradient allowed to flow into the sedimentation chamber C. The stopcock E was then closed and the column allowed to stand for 45 minutes. The column was then fractionated by opening the stopcock and collecting the effluent as 10ml. volumes into a numbered series of polystyrene universal containers. The containers were centrifuged at 400g for 20 minutes and the supernatant discarded. A smear was prepared from each cell deposit using a series of numbered slides and stained by Papanicoloau's method as described in Section 3.1.2. The stained smears were examined at x 600 magnification and the cell types present in each numbered smear were recorded.

6.5.3 Results

Microscopy showed that lymphocytes and erythrocytes spanned the BSA gradient from 2.0% to 1.45%. Neutrophils first appeared at 1.7% and disappeared at 1.15%. The

Figure 28 Apparatus used for velocity sedimentation separation of mixed-cell suspension

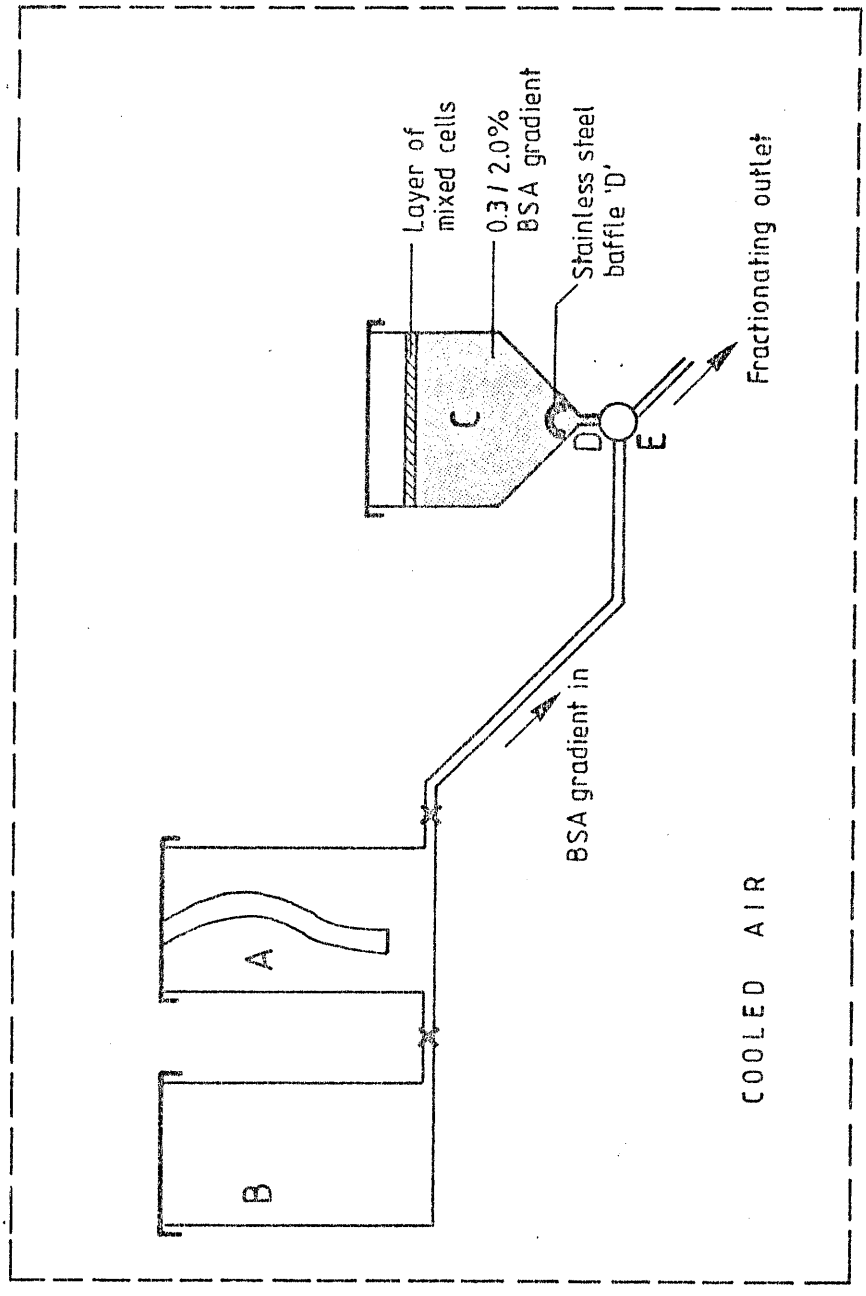
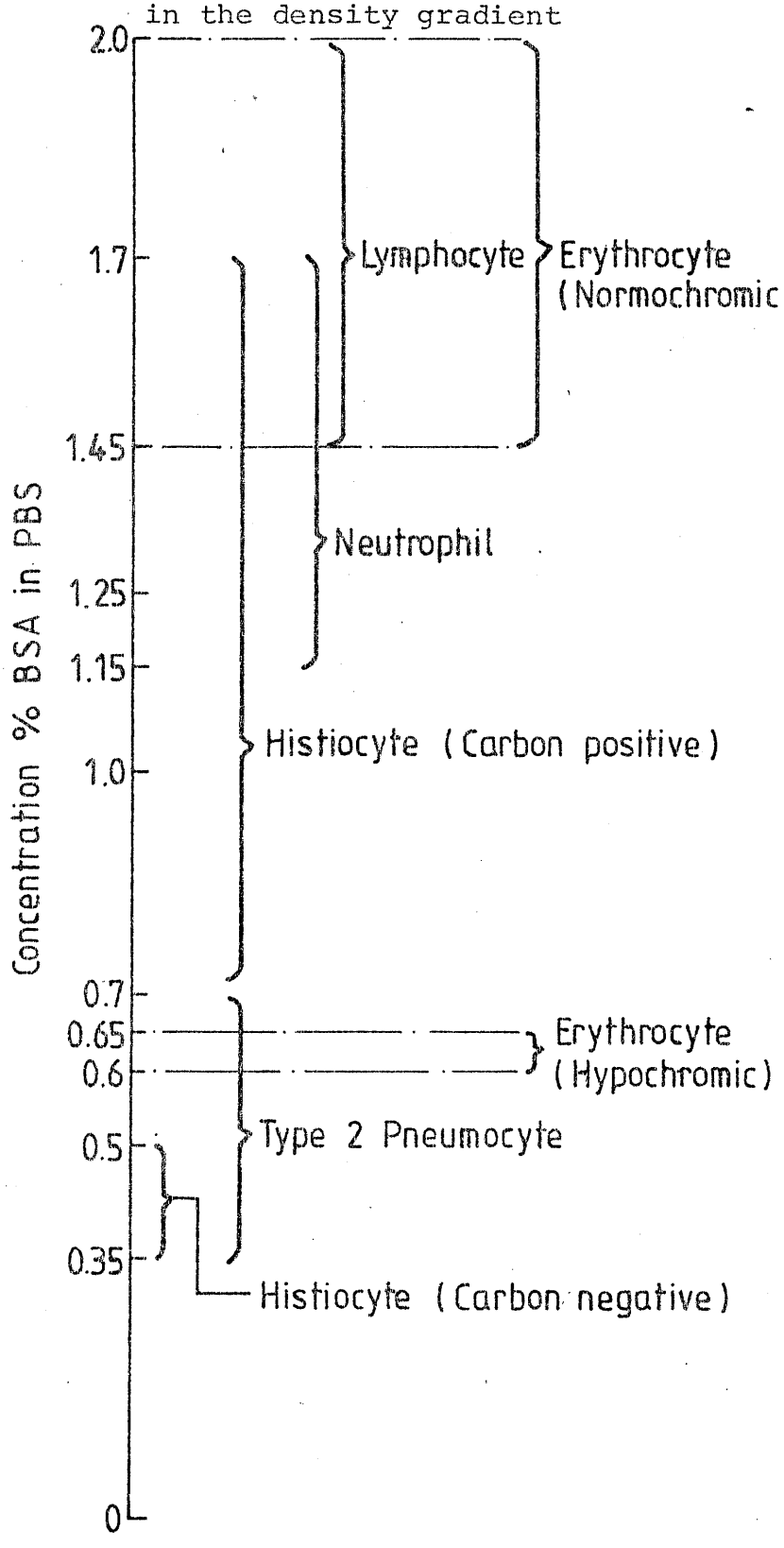


Figure 29 The distribution of the cell content of bronchial washings after settling for 45 minutes in the density gradient



highest concentration of neutrophils occurred at 1.25% BSA. Carbon-containing histiocytes also appeared at 1.7% and disappeared after 0.7% BSA. The highest concentration of histiocytes with carbon was found at 1.0% BSA. Hypochromic erythrocytes were found between 0.65% and 0.6% BSA. Type 2 pneumocytes appeared at 0.7% and disappeared at 0.35% BSA: most were found at 0.5% BSA. Histiocytes without endocytosed carbon were found between 0.5% and 0.35% BSA.

These results are shown in diagram form in Figure 29.

6.5.4 Discussion

The technique separates carbon-free histiocytes from carbon-containing histiocytes. It can be seen from Figure 29 that whereas the concentration range of bovine serum albumin over which carbon-free histiocytes are found is restricted to 0.15% (from 0.35 to 0.50% BSA) carbon-containing histiocytes span a concentration range of 1.0% (from 0.7 to 1.7% BSA). This probably reflects the level of phagocytic activity of the histiocytes. Although counts of carbon particles in the histiocyte cytoplasm were not done, the cells with the greatest carbon load were present in the higher concentration of bovine serum albumin suggesting that the greater the carbon load in the cytoplasm, the greater is the velocity of the histiocyte in the test conditions.

It is proposed to investigate this by harvesting the histiocytes obtained from the 0.7 to 1.7% BSA concentration range and to subject this fraction to further separation. This will be done by constructing the 0.7 to 1.7% BSA gradient over the total depth of the sedimentation column thus

enhancing the velocity differences between the cells. If successful, the method can be applied to bronchial washings from individuals exposed to chrysotile and it should be possible to separate the histiocyte population into carbon-free, carbon-containing and carbon+chrysotile containing classes. This will enable the cytochemistry and subcellular structure of the individual classes of human histiocytes to be studied.

CHAPTER SEVEN

General Discussion

General Discussion

Recently, Selikoff and Lee (1978), with the assistance of eighteen collaborators, published a comprehensive survey of published research on asbestos and disease. It can be seen from this extensive review that no conclusive evidence has yet emerged concerning the way retained chrysotile fibre is transferred from the airway to the lung parenchyma or why exposure to such fibre induces lung fibrosis in some individuals and not in others.

It is certain that, before coming into contact with cells, inhaled chrysotile has been exposed to respiratory tract secretions. A knowledge of the subsequent state of the retained fibre would seem essential to a proper interpretation of experimental systems concerned with human cell response to chrysotile. For this reason the current research has investigated *in vitro* the likely interactions between chrysotile and the human airway mucosa. The primary aim was to reconstruct events that probably occur at the air/mucus interface and to look for indicators that might serve to predict fibrogenesis in the human lung after exposure to chrysotile dust. Accordingly, answers were sought to four questions.

1. The first question was: Does inhalation of chrysotile dust bring about changes in the proteins of the airway secretions?

Differences in protein content were found between sputa produced by unexposed and chrysotile-exposed donors. The findings, discussed in detail in Chapter 2, indicate that chrysotile probably induces hypersecretion resulting in the production of sputum lower in total protein concentration than that produced in the absence of chrysotile dust. Once the

process of lung fibrosis has been established, the stimulus to hypersecretion is removed or occluded. Although analysis of serial sputum specimens from an exposed donor is unlikely to reveal the fall in total protein, a comparison of the concentrations of individual electrophoretic fractions with total protein should show significant correlation, $p = 0.05$, for all fractions except beta/gamma globulin when the sputum is from an exposed donor without asbestosis. By contrast, sputum from donors with asbestosis should show correlation only between beta/gamma globulin and total protein. However, the lysozyme fraction has to be excluded from this general statement because it was not detected in about 66% of sputa although lysozyme concentration was found to correlate with total protein, $p = 0.05$, within the donor group 'exposed without asbestosis'.

Immunodiffusion analysis showed significant differences. $p = 0.05$, between donor groups for three specific proteins; transferrin, gamma a and gamma g globulins and these are discussed in Section 2.4.2.1. However, after adsorption of sputum proteins by chrysotile, some of the immunoprecipitates were of greater diameter than those obtained from the untreated sputum. The immunodiffusion results for transferrin are particularly noteworthy because of its role in the binding and transportation of iron and suggest that serum transferrin activity in asbestosis merits further investigation.

A non-serous glycoprotein with $beta_3$ electromobility was isolated by cation exchange fractionation. Part of this protein probably complexes with a serum gamma globulin and the frequency of detection in sputum of this complex declines as fibrosis progresses.

2. The second question was: Does inhaled chrysotile cause a characteristic cell response in the human respiratory tract?

The study shows that chrysotile causes damage to the epithelium of the middle and distal airways. The fact that metaplastic cells were seen three times more frequently in sputum from donors with asbestosis than other exposed donors suggests that more extensive areas of the mucosa are damaged when asbestosis is present. By contrast, evidence of damage to the distal airway epithelium was found with equal frequency in both exposed donor groups with a 50% chance of detection in a particular sputum specimen. This may be a sequel to the hypersecretion induced by the inhalation of chrysotile. Eventually, accumulation of the increased secretion triggers the cough reflex and it is likely that during coughing, some retained chrysotile is driven into the alveoli. The high detection rate of Type 1 and Type 2 pneumocytes in both exposed groups of donors indicates that chrysotile in the alveolus probably causes repeated cell loss from the alveolar epithelium in both fibrosing and non-fibrosing lungs. The observation also implies than an affected alveolus is not necessarily blocked by cells and secretion. The presence of iron-containing histiocytes in the sputum from donors in both groups exposed to chrysotile is probably associated with the iron/protein complex found in asbestos bodies. The findings also show that if the histiocytes contain endocytosed chrysotile or asbestos bodies fibrosis is probably present in the lungs.

3. The third questions posed was: Is there a general pattern of protein adsorption onto inhaled chrysotile from respiratory tract secretion?

The results from the present study clearly show that chrysotile fibre first takes up water then protein when immersed in sputum. The chrysotile/sputum system reached a steady state between thirty minutes and two hours after the addition of the sputum. Interchange of proteins probably occurred at the sputum/chrysotile interface during the first thirty minutes but when equilibrium was reached, a general pattern of protein adsorption was found. The ratio of the mean weights of adsorbed protein from the fractions was 1:1:3:1; albumin:alpha globulin:beta/gamma globulin:lysozyme and is roughly in the proportion of the monomeric molecular weights of proteins. This consistency of pattern in protein uptake suggests that the protein matrix on the fibre surface has a regular form. The weight of protein adsorbed by chrysotile was about 45 mg protein/g chrysotile.

4. The fourth question was: What effect does chrysotile coated with sputum proteins have on human cells, particularly the lung histiocytes?

When chrysotile is coated with proteins adsorbed from sputum, human leucocytes are protected from the cytolytic property of uncoated chrysotile for at least the first sixty minutes of exposure. It is only after 18 hours of exposure that cell survival (as judged by cell counts and viability) is impaired indicating that coated chrysotile possesses latent cytotoxicity.

Progress on this section of the study was hindered by

the problems associated with the isolation of human lung histiocytes. See discussion, Section 6.1.3, but the initial findings given in Section 6.4.2 suggest that human lung histiocytes are similarly affected when exposed to chrysotile coated with sputum proteins.

These findings can be interpreted in the context of the pathophysiology of inhaled chrysotile as follows.

When chrysotile dust is carried in the current of inhaled air, the period of buoyancy (1.74 seconds) is too brief for a significant amount of water to be taken up by the fibre. On making contact with the mucosa, chrysotile immediately absorbs water and subsequently adsorbs proteins from the secretion. During the initial phase of protein adsorption, interchange of proteins may take place at the interface between chrysotile and secretion. If this does occur, it can be accounted for by the surface characteristics of chrysotile fibre. Pundsack (1955) showed that chrysotile can be regarded as a magnesium hydroxide layer on a silicate substrate. When the fibres are suspended in water, the surface of the chrysotile is negatively charged, but in the presence of water containing carbon dioxide, the surface becomes positively charged. When chrysotile is immersed in sputum, the initial rapid uptake of water could result in a temporary net negative charge on the surface that is ultimately reversed in the presence of the carbon dioxide rich secretion. The intervening period of instability of surface charge would account for the fluctuation found in the proportions of protein fractions taken up from the sputum and also why the fibre exhibits avidity for no particular protein. However, when the steady state is reached, the total weight of protein

taken up is 44.8 ± 7.7 mg protein/g chrysotile. Morgan (1974) found that chrysotile with similar surface area (22.4m^2/g) took up 45 mg human albumin/g chrysotile. He considered that this constituted a monolayer on the fibre surface. This suggests that, in the current study, the protein matrix would be uneven in thickness and probably not a coherent film. If this is the case, it accounts for the finding that fibre coated with sputum proteins retained cytotoxic properties albeit much attenuated.

The cytological results suggest that part of the retained chrysotile reaches the lung alveoli and causes damage to the epithelium. Type 1 pneumocytes are shed causing proliferation of Type 2 cells. This accords with the histological description given by Kuhn and Kuo (1973) of alveolar epithelium reacting to injury by chrysotile.

At this stage of insult by chrysotile the cytological and biochemical findings taken together imply that the alveolar ducts containing chrysotile are not tightly sealed off and the capillary endothelium has not been breached. This is the state of the lungs of donors exposed to chrysotile without asbestosis. Iron mobilisation is taking place as judged by raised transferrin in sputum and histiocytes containing ferric iron and is probably associated with asbestos body formation. The finding of an increased frequency of detection of lymphocytes in sputum from exposed donors contrasts with the neutrophil response in guinea pigs reported by Schoenberger et al (1981) and Schoenberger et al (1982). The cytological findings in the current study, however, show differences only in histiocytes between donors without and

with fibrosis. Histiocytes with endocytosed chrysotile or asbestos bodies and the increased frequency of extracellular asbestos bodies in sputa from patients with asbestosis may indicate that these donors possess greater accumulation of retained chrysotile than donors without asbestosis.

Keogh and Crystal (1982) have described alveolitis as the key to interstitial lung disorders. It would appear that further study of the cells and retained fibre content of human alveoli in asbestosis by means of bronchial lavage is a necessary step toward an understanding of human asbestosis.

Supplement

1) Smoking

At the planning stage of my study, the possible effect of smoking on the respiratory tract epithelium and its secretions was, of course, considered. Accordingly, for each investigation, the sputum donors were matched for smoking habit between the three donor classes. This strategy reduced the complexity of experimental design and enabled the effect of smoking to be discounted when interpreting significant differences in results between the three donor classes.

2) Controls

Individuals comprising the control group of donors unexposed to asbestos were drawn from personnel working in clean-air premises such as offices and the Industrial Health Unit. These places are located remote from production areas and regular monitoring of air in these clean areas yields counts of less than 0.1 particle/ml air.

Members of the group 'unexposed to asbestos' had no domestic contact with workers exposed to asbestos. However, casual exposure to various atmospheric pollutants other than asbestos could have occurred to any or all of the sputum donors.

3) Collection of Sputum Specimens - see 3.1.1

Early morning sputum was not used for the investigation because of the likelihood of inspissatory changes taking place in secretions retained overnight in the airways. The principal changes that might occur in these circumstances

are concentration of extracellular protein and damage to exfoliated epithelial cells and migrant cells such as histiocytes and neutrophils.

The sputum donors were examined in the Industrial Health Unit-a building remote from the factory complex.
To ensure that sputum originated in the middle and distal airways, the donors undertook a water mouthwash and gargle before being subjected to physiotherapy to the chest and back. The individual was then asked to attempt the 'deep cough' technique, that is exhalation of tidal air followed immediately with further forced exhalation of the expiratory reserve volume, see figure 4 on page 14. This procedure induced a bout of coughing resulting in a yield of secretion (0.6ml to 2.0ml) in 87% of the panel. The sputum produced was passed directly into a sterile polystyrene (60ml) container (Sterilin Ltd., Teddington).

4) Identification of Certain Cells

This aspect of the study is the subject of continuing research.

The aim of the current work is to isolate and characterise the type 2 pneumocyte by extending the cell fractionation reported in Chapter 6. A technique is being developed using a cell sorting system CS 1001, PHYWE A.G. Gottingen (Camlab Ltd., Cambridge). The cell fractions will be tested for cytokeratin using a monoclonal antibody/ peroxidase staining method (Labsystems (U.K.) Ltd., Uxbridge).

Cytokeratin is an intermediate cell filament substance present specifically in all epithelial cells.

This further research is being carried out with financial support from the Yorkshire Regional Health Authority, Regional Research Committee, Project reference B 2, under the general guidance of Professor T.G. Baker, University of Bradford, using bronchial washings supplied by Dr. D.A.G. Newton, Consultant Chest Physician, Bradford Hospitals.

5) Non-Specific Dust Effect

This study has investigated the effects of asbestos in the human respiratory tract. Further extensive work would be required to demonstrate whether any of the findings is a response specific to asbestos exposure. However, the same experimental approach can be used to investigate the consequences of inhaling other types of dust e.g. coal dust. Of particular interest, however, would be the result obtained by re-working the investigations reported in Chapters 5 and 6 but substituting alternative dusts. The protein fractions adsorbed from respiratory secretions is probably largely dependent upon the physico-chemical characteristics of a particular dust - especially the nature and density of charge at the surface of inhaled particles. These same properties will act as primary determinants governing the cellular response particularly with respect to cytotoxicity and phagocytosis.

REFERENCES

References

Ackerman, S.K., Tuckerman, S.H., Douglas, S.D. (1981). Isolation and Culture of Human Blood Monocytes in Investigation of Phagocytes in Disease. (Douglas S.D., Quie P.G eds), Edinburgh; Chruchill Livingstone: 66-75.

Adamson, I.Y.R., Bowden, D.H. (1971). Sialomucin and Pulmonary Cell Injury. Arch Pathol: 92, 273-278.

Alli..son, A.C. (1970). Effects of Silica and Asbestos on Cells in Culture in Inhaled Particles and Vapours. Oxford: Pergamon Press: 3, (1), 437-445.

Alli son, A.C. (1973). Experimental Methods in Cell and Tissue Culture, Effects of Asbestos Particles on Macrophages, Mesothelial Cells and Fibroblasts in Biological Effects of Asbestos: Lyon: International Agency for Research on Cancer, 89-93.

Anderson, W.A.S. (1966). Pathology: London: Henry Kimpton.

Annotation (1973). Small Particles in Small Airways. Lancet: 2,948-949.

Asbestos Resultations (1931), Statutory Rules and Orders, 1931, No. 1140. London: HMSO.

Ashcroft, T., Heppleston, A.G. (1973). The Optical and Electron Microscopic Determination of Pulmonary Asbestos Fibre Concentration and its Relation to Human Pathological Reaction, J. Clin, Pathol: 26, 224-234.

Atassi, M.Z., Barker, S.A., Houghton, L.E., Mullard, K.S. (1961). Mucoproteins in Bronchial Mucus. Nature, 192, 1269-1270.

Atassi, M.Z., Barker, S.A., Stacey, M. (1962). Mucoproteins in Bronchial Mucus. Clin. Chim. Acta. 7, 588-595.

Bernstein, J., Yang, S.S., Hahn, H.S., Kikkawa, Y. (1969). Mucopolysaccharides in the Pulmonary Alveolus. Lab. Invest. 21(5), 420-425.

Beverley, W.H.A. (1973). A Study of Discernable Changes in a Group of Asbestotics Before and After Certification. J. Sec. Occup. Med. 23, 61-65.

Biserte, G., Havez, R., Culvier, R. (1965). Etudes des Glycopeptides du Mucus Fibrillaire de la Secretion Bronchique. Exposes Ann Biochem. Med. 24, 85-91.

Blake, J. (1973). A Note on Mucus Shear Rates. Resp. Physiol. 17, 394-399.

Blount, M. Holt, P.F., Leach, A.A. (1966). The Protein Coating of Asbestos Bodies. Biochem. J. 101, 204-207.

Boat, T.F., Cheng, P.W., Iyer, R.N., Carlson, D.M., Polony, I. (1976). Mucous Glycoproteins of Nonpurulent Tracheobronchial Secretion and Sputum of Patients with Bronchitis and Cystic Fibrosis. Arch. Biochem. Biophys. 177, 95-104.

Bowden, D.H. (1981). Alveolar Response to Injury. Thorax, 36, 801-804.

Bowes, D., Corrin, B. (1977). Ultrastructural Immunocytochemical Localisation of Lysozyme in Human Bronchial Glands. Thorax, 32, 163-170.

Boyum, A. (1968). Isolation of Mononuclear Cells and Granulocytes from Human Blood. Scand. J. Clin. Lab. Invest. (Suppl): 97, (21), 77-89.

Brand, E., Kassell, B. Saidel, L.J. (1944). Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation - 3 amino acid composition of plasma proteins. J. Clin. Invest. 23, 437-451.

Brewis, R.A.L. (1975). Lecture Notes on Respiratory Disease. Oxford, Blackwell Scientific Publications.

Brogan, T.D. (1960). The High Molecular Weight Components of Sputum. Br. J. Exp. Path. 41, 288-297.

Brogan, T.D., Ryley, H.C., Allen, L., Hutt, H. (1971). Relation Between Sputum Sol Phase Composition and Diagnosis in Chronic Chest Diseases. Thorax: 26, 418-423.

Buchanan, W.D. (1979). Asbestos-Related Diseases Part 1 Introduction in Asbestos (Michaels L., Chissick, S.S. eds). Chichester, John Wiley and Sons, 395-408.

Bukantz, S.C., Berns, A.W. (1958). Mucus in Status Asthmaticus. J. Allergy, 29, 29-34.

Butler, M.G. (1973). A New Method for the Identification of Acid Glycoproteins. Med. Lab. Tech. 30, 105-112.

Capel, L.H. (1979). Asbestos-Related Disease, Part 3, Asbestos Associated Thoracic Disorders, Clinical Features. In Asbestos (Michaels, L., Chissick, S.S. eds). Chichester, John Wiley and Sons, 457-464.

Carr, I., Hancock, B.W., Ward, A.M. (1977). Lymphoreticular Disease: Oxford, Blackwell Scientific Publications, 7-9.

Carter, G.S. (1931). Aquatic and Aerial Respiration in Animals, Biol. Rev. 6, 1-35.

Chamberlain, D.W., Nopajaroonsri, C., Simon, G.T. (1973). Ultrastructure of the Pulmonary Lymphoid Tissue. Am. Rev. Resp. Dis. 108, 621-663.

Clamp, J.R. (1977). The Relationship Between Secretory Immunoglobulin A and Mucus. Biochem. Soc. Trans. 5, (5), 1579-1581.

Cooke, W.E. (1927). Pulmonary Asbestosis. Br. Med. J. 2, 1024-1025.

Creeth, J.M. (1978). Constituents of Mucus and Their Separation, Br. Med. Bull. 34, (1), 17-24.

Currie, G.A., Hedley, D.W. (1977). Monocytes and Macrophages in Malignant Melanoma, Peripheral Blood Macrophages Precursors. Br. J. Cancer, 36, 1-6.

Degand, P., Roussel, P., Lamblin, G. (1974). Definition des Mucines Synthetises au Niveau des Kystes Bronchogeniques. Clin. Chim. Acta. 50, 223-236.

Dent, R.G., Cole, P. (181). In vitro Monocyte Maturation as a Prediction of Survival in Sqamous Carcinoma of the Lung. Thorax, 36, 446-451.

Desai, R., Tetley, T.D., Curtis, C.G., Powell, G.M., Richard, R.J (1978). Studies on the Fate of Pulmonary Surfactant in the Lung, Biochem. J. 176, 455-462.

Desai, R., Hext, P., Richards, R. (1975). The Prevention of Asbestos Induced Haemolysis. Life Sciences: 16, 1931-1938.

Drury, R.A.B., Wallington, E.A. (1967). Perl's Prussian Blue Method for Haemosiderin. In Carleton's Histological Technique, 4th edn. London: Oxford University Press, 225.

Farrand, S.B. (1970). The Feulgen Reaction In Introduction to Quantitative Cytochemistry (Wied, G., Bahn, G.F. eds), New York, Academic Press.

Ferrin, J., Urbankova, G., Vickova, A. (1965). Pulmonary Clearance and the Function of Macrophages. Arch. Environ. Health, 10, 790-796.

Van Furth, R., Conn, Z.A., Hirsch, J.G., Humphrey, J.H., Spector, W.G., Langevoort, H.G. (1972). The Mononuclear Phagocyte System; a New Classification of Macrophages Monocytes and their Precursor Cells. Bull Wld. Hlth. Org. 46, 845-852.

Goldman, J.M. (1972). Tutorials in Postgraduate Medicine Haematology (Hoffbrand, A.B., Lewis, S.M. eds) London, Wm. Heinemann Medical Books Ltd., 426-432.

Gross, P., Davis, J.M.G., Harley, R.A., de Treville, R.T.P. (1973) Lymphatic Transport of Fibrous Dust from the Lungs. J. Occup. Med. 15 (3), 186-189.

Harington, J.S., Miller, K. MacNab, G. (1971). Haemolysis by Asbestos: Environ. Res. 4, 95-117.

Harris, R.K., Fraser, D.A. (1976). A Model for the Deposition of Fibres in the Human Respiratory System. Am Ind. Hyg. Assoc. J. 37, 73-76.

Havez, R., Roussel, P., Degand, P., Randoux, A., Biserte, G. (1967). Etude des structure fibrillaire de la secretion bronique humaine. Clin. Chim. Acta. 17, 281-295.

Heppleston, AG. (1963) (a) Deposition and Disposal of Inhaled Dust. Arch. Environ. Hlth. 7, 548-555.

Heppleston, A.G. (1963) (b) The Disposal of Inhaled Particulate Matter - a Unifying Hypothesis. Am. J. Pathol. 42, (2), 119-135.

Hodgson, A.A. (1965). Fibrous Silicates Lecture Series No. 4, London: Royal Institute of Chemistry.

Hodgson, A.A. (1979). Chemistry and Physics of Asbestos. In Asbestos (Michaels, L., Chissick, S.S. eds). Chichester, John Wiley and Sons, 67-114.

Horsfield, K. (1971). Models of the Human Bronchial Tree. J. Appl. Physiol. 31, 207-211.

House, W. (1964). Toxicity of Cell Culture Medium Due to Filtration Through Asbestos Pads. Nature, 201, 1242.

Hunt, R.D. (1965). Routine Lung Function Studies on 630 Employees in an Asbestos Processing Factory. Ann. N.Y. Acad. Sci. 132, 406-420.

Hunter, D. (1974). The Diseases of Occupations 5th edn. London: English Universities Press, 972.

Itzhaki, R.F., Gill, D.M. (1964). Estimation of Serum Proteins. Anal. Biochem, 9, 401-412.

Jones, R. (1978). The Glycoproteins of Secretory Cells in Airway Epithelium. In Respiratory Tract Mucus (Porter, R., Rivers, J., O'Connor, M. eds). Amsterdam, Elsevier Excerpta Medica North Holland: 175-193.

Jordan, B.M., Baker, J.R. (1955). A Modified Unna Pappenheim Stain. Quart. J. Micr. Sci. 96, 177-179.

Kapanci, Y., Assimacopoulos, A., Irle, C., Zwahlen, A., Gabbiani, G. (1974). 'Contractile Interstitial Cells' in Pulmonary Alveolar Septa. A Possible Regulator of Ventilation/Perfusion Ratio. J. Cell. Biol. 60, 375-392.

Keogh, B.A., Crystal, R.G. (1982). Alveolitis the key to interstitial lung disorders. Thorax. 37, 1-10.

Kikkawa, Y, Yoneda, K. (1973). Isolation of Type 2 epithelial cells from rat lung. Am. Rev. Resp. Dis. 107-1115-1124.

Kilburn, K.H. (1968). A Hypothesis for Pulmonary Clearance and its Implications. Am. Rev. Resp. Dis. 98, 449-463.

Kimura, K. (1970). Experimental Studies on the Aerosol Deposition in the Human Respiratory System. J. Sci. Labour, 47, (6), 373-382.

Klockars, M., Reitamo, S. (1975). Tissue Distribution of Lysozyme in Man. J.J. Histochem. Cytochem. 23, (12), 932-940.

Kohler, H. (1968). Normal Bronchial Secretion. Z. Tuberk, 129, 261-274.

Korhonen, L.K., Hopainen, E., Paavolainen, M. (1969). Some Histochemical Characteristics of the Tracheobronchial Tree and Pulmonary Neoplasms. Acta Histochem. 32, 57-73.

Kuhn, C., Kue, T.T. (1973). Cytoplasmic Hyaline in Asbestosis. Arch. Pathol. 95, 190-194.

Lamb, D. (1968). Intracellular Development and Secretion of Mucus in the Normal and Morbid Bronchial Tree. Ph.D. thesis, University of London.

Lamb, D., Reid, L. (1969). Histochemical Types of Acidic Glycoprotein Produced by the Mucous Cells of the Tracheobronchial glands in man. J. Pathol. 98, 213-229.

Lamb, D., Reid, L. (1970). Histochemical and Autoradiographic Investigation of the Serous Cells of the Human Bronchial Glands, J. Pathol. 100, 127-138.

Laval, P., Meyer, G., Rosier, M. (1961). Electrophorese et immunoelectrophorese des secretions bronchiques. J. Franc. Med. Chir. Thor., 15, 583-593.

Lorentz, T.H., Korst, D.R., Simpson, J.F., Musser, M.J. (1957). A Quantitative Method for Lysozyme Determination. J. Lab. Clin. Med. 49, 145-150.

McCarthy, C., Reid, L. (1964). Intracellular Mucopolysaccharides in the Normal Human Bronchial Tree. Quart. J. Exp. Physiol. 49, 85-94.

McDonald, S. (1927). Histology of Pulmonary Asbestosis. Br. Med. J. 2, 1025-1026.

McMillan, G.H.G., Sheers, G., Pethybridge, R. (1978). A Radiological Follow up of the Effects of Asbestos in Dock-yard workers in Devonport. J. Roy Naval Med. Serv. 64, 88-104.

MacNab, G. Harington, J.S. (1967). Haemolytic Activity of Asbestos and Other Mineral Dusts. Nature, 214, 522-523.

McVittie, J.C. (1965). Asbestosis in Great Britain. Ann N.Y. Acad. Sci., 132, 128-138.

Masson, P.L., Heremans, J.F., Prignott, J.J., Wauters, G. (1966). Immunohistochemical Localisation and Bacterio-static Properties of an Iron Binding Protein from Bronchial Mucus. Thorax, 21, 538-544.

Matthew, K.W., Spector, S., Lemm, J., Heremans, J.F. (1966). Bronchial Secretion After Laryngectomy. Biochem Biophys Acta. 120, 172-184.

Matthew, J.L., Martin, J.H. (1971). Atlas of Human Histology and Ultrastructure. Philadelphia. Lea and Fabiger, 282.

Maximow, A.A., Bloom, W. (1948). Textbook of Histology. Philadelphia. W.B. Saunders.

Merewether, E.R.A., Price, C.W. (1930). Report on the Effects of Asbestos Dust on the Lungs and Dust Suppression in the Asbestos Industry. London, HMSO.

Miller, R.G., Phillips, R.A. (1969). Separation of Cell by Velocity Sedimentation. J. Cell. Physiol. 73, 191-201.

Millipore, (UK). 'PhoroSlide' electrophoresis sytem. London. Millipore, (UK).

Medin, B.E., Greenberg, S.D., Buffler, P.A., Lockhart, J.A., Seltzman, L.H., Awe, R.J. (1982). Asbestos Bodies in a General Hospital Population. Acta. Cytol. 26(5), 667-670.

Moore, M.A.S. (1978). Regulatory Role of the Macrophage in Haemopoesis. In Stem Cells and Tissue Homeostasis. (Lord,B.I., Potten, C.S., Cole, R.J. eds). Cambridge: Cambridge University Press, 187-202.

Morgan, A. (1974). Adsorption of Human Serum Albumin by Asbestiform Minerals and Its Application to the Measurement of Surface Areas of Dispersed Samples of Chrysotile. Environ. Res. 7, 330-341.

Morgan, J.F., Morton, H.J., Parker, R.C. (1950). A Synthetic Tissue Culture Medium. Proc. Soc. Exp. Biol. Med. 73, 1-15.

Murphy, R.L.H. (1978). Diagnosis of 'asbestosis' Observations from a Longitudinal Survey of Shipyard Pipe Coverers. Am. J. Med. 65, (3), 488-498.

Murray, M. (1907). Departmental Commission on Compensation for Industrial Diseases. Cmd 3495:14, Cmd 3496: 127-128. London, HMSO.

Myrvik, Q.M. (1973). The Role of the Alveolar Macrophage. J. Occup. Med. 15(3), 190-193.

Pearse, A.G.E. (1972). Histochemistry Theoretical and Applied. London, J and A. Chruchill Ltd.

Pump, K.K. (1974). Fenestrae in the Alveolar Membrane of the Human Lung. Chest, 65(4), 431-436.

Pundsack, F.L. (1955). The Properties of Asbestos. J. Physiol. Chem. 59, 892-895.

Reid, L., Clamp, J.R. (1978). Biochemical and Histochemical Nomenclature of Mucus. Br. Med. Bull. 34, (1), 5-8.

Roberts, G.P. (1974). Isolation and Characterisation of Glycoproteins from Sputum. Eur. J. Biochem. 50, 265-280.

Roberts, G.P. (1976). Fractionation and Comparison of Protein from Bovine Uterine Fluid and Bovine Allantoic Fluid. Arch. Biochem. Biophys. 173, 528-537.

Pola-Pleszczynski, M., Churchill, W.H. (1978). Purification of Human Monocytes by Continuous Gradient Sedimentation in Ficoll. J. Immunol. Methods. 20, 255-262.

Ryley, H.C. (1970). An Electrophoretic Study of the Sol Phase of Sputum. J. Lab. Clin. Med. 75, 382-390.

Ryley, H.C., Brogan, T.D. (1968). Variation in the Composition of Sputum in Chronic Chest Disease. Br. J. Exp. Pathol. 49, 625-633.

Schneeberger, E.E. (1976). Ultrastructural basis for Alveolar-Capillary Permeability to Protein. In Lung Liquids (Porter, R., O'Connor, M. eds). Amsterdam, Elsevier Excerpta Medica North Holland, 3-27.

Schnitzer, R.J., Pundsack, F.L. (1970). Asbestos Haemolysis. Environ. Res. 3, 1-13.

Schoenberger, C.I., Hunninghake, G.W., Gadek, J., Crystal, R.G. (1981). Inflammation and Asbestos, Characterisation and Maintenance of Alveolitis following Acute Exposure. Chest (Suppl). 80, 70-71S.

Schoenberger, C.I., Hunninghake, G.W., Kawanami, O., Ferrans, V.J., Crystal, R.G. (1982). Role of Alveolar Macrophages in Asbestosis, Modulation of Neutrophil Migration to the Lung after Acute Asbestos Exposure. Thorax, 37, 803-809.

Schulze, A.L, Heremans, J.F. (1966). Molecular Biology of Human Proteins. Amsterdam. Elsevier North Holland, 816-831.

Seiler, H.E., (1928). A Case of Pneumoconiosis Result of Inhaling Asbestos Dust. Br. Med. J. 2, 982-983.

Selikoff, I.J., Lee, D.H.K. (1978). Asbestos and Disease, New York. Academic Press, 145,

Shah, R.O., Dye, W.E. (1966). Use of Dithiothreitol to Replace N-Acetyl-l-Cysteine for Routine Sputum Digestions Decontamination for the Culture of Mycobacteria. Am. Rev. Resp. Dis. 94, 454-458.

Sluis-Cremer, G.K. (1970). Asbestosis in South African Miners. Environ. Res. 3, (4), 310-319.

Spicer, S.S., Chakrin, L.W., Wardell, J.R., Kendrick, W. (1971). Histochemistry of Mucosubstances in the Canine and Human Respiratory Tract. Lab. Invest. 25, (6), 483-490.

Staub, N.C., Gee, M., Vreim, C. (1976). Mechanisms of Alveolar Flooding in Acute Pulmonary Oedema. In Lung Liquids (Porter, R., O'Connor, M. eds). Amsterdam, Elsevier Excerpta Medica North Holland, 255-272.

Steiner, R.F. (1953). Reversible Association Processes of Globular Proteins Fluorescence Methods in Studying Protein Interactions. Arch. Biochem. Biphys. 46, 291-311.

Stockley, R.A., Burnett, D. (180). Local Ig A Production in Patients with Chronic Bronchitis, Effect of Acute Respiratory Infection. Thorax, 35, 202-206.

Strang, L.B. (1978). The Permeability of Lung Capillary and Alveolar Walls as Determinants of Liquid Movements in the Lung. In Lung Liquids (Porter, R., O'Connor, M. eds). Amsterdam, Elsevier Excerpta Medica, North Holland, 49.64.

Taylor, R.G.W. (1967). Practical Cytology. London: Academic Press.

Thomas, P.A. (1972). Pulmonary Surfactant and Histopathological Observations from a Study of 106 Patients. J. Surg. Res. 13, (1), 1-6.

Teirstein, A.S. (1978). Respiratory Portal of Entry. In Asbestos (Selikoff, I.J., Lee, D.H.K., eds). New York. Academic Press, 339-355.

Tubbs, R.R., Benjamin, S.P., Osborne, D.G., Barenberg, S. (1978). Surface and Transmission Characteristics of Desquamative Interstitial Pneumonitis. Hum. Pathol. 9, (6), 695-703.

Warfinge, L.E. (1955). Paper Electrophoresis of Sputum in Bronchiolar Carcinoma. Acta. Med. Scand. 153, 695-703.

Weibel, E.R. (1963). Morphometry of the Human Lung, New York. Academic Press.

Weiss, W. (1971). Cigarette Smoking Asbestos and Pulmonary Fibrosis. Am. Rev. Resp. Dis. 104, 223-227.

Whayne, T.F., Severinghause, J.W. (1968). Experimental Hypoxic Pulmonary Oedema in the Rat. J. Appl. Physiol. 25, 729-736.

Whitaker, A.M. (1975). Subculture of Cell Line. In Laboratory Manual of Cell Biology (Hall, D., Hawkins, S. eds). London, English Universities Press, 4-7.

Whitby, L.E.H., Britton, C.J.C. (1953). Disorders of the Blood. London: J and A Churchill.

Widdicombe, J.G. (1978). Control of Secretion of Tracheo-bronchial Mucus. Br. Med. Bull. 34, 57-61.

Wu, H. (1922). A New Colorimetric Method for the Determination of Plasma Proteins. J. Biol. Chem. 51, 33-39.

Zenker, (1867). Iron Lung Siderosis Pulmonum. Dtsch. Arch. Klin. Med., 2, 116.

Zielhuis, R.K. (1977). Public Health Risks of Exposure to Asbestos. Commission for the European Communities, Oxford, Pergamon Press.